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Epidemiology of *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans* in Belgian cystic fibrosis patients, relying on molecular typing techniques

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"Live as if you were to die tomorrow.

Learn as if you were to live forever,"

Gandhi (1869-1948)

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List of abbreviations:

ABPA: allergic bronchopulmonary aspergillosis

AFLP: amplified fragment length polymorphism

AP-PCR: arbitrarily primed polymerase chain reaction

ASL: airway surface layer

ATP: adenosine triphosphate

A. xylosoxidans: *Achromobacter xylosoxidans*

B. cepacia: *Burkholderia cepacia*

cAMP: cyclic adenosine monophosphate

CBAVD: congenital bilateral absence of vas deferens

CF: cystic fibrosis

CFP: cystic fibrosis phenotype

CFTR: cystic fibrosis transmembrane conductance regulator

DIOS: distal intestinal obstruction syndrome

DNA: deoxyribonucleic acid

EFA: essential fatty acids

ELISA: enzym-linked immunosorbent assay

EnaC: epithelial sodium channel

fAFLP: fluorescent amplified fragment length polymorphism

HCW: health care worker

H. influenzae: *Haemophilus influenzae*

IL: interleukine

LPS: lipopolysaccharide

MRSA: Methicilline resistant *Staphylococcus aureus*

NF-κβ: nuclear factor κβ

OP culture: oropharyngeal culture

P. aeruginosa: *Pseudomonas aeruginosa*

PCL: periciliary liquid layer

PCR: polymerase chain reaction

PFGE: pulsed field gel electrophoresis

RAPD: random amplification of polymorphic DNA

Rehab A/B: rehabilitation centre A/B

RNA: ribonucleic acid

RSV: respiratory syncytial virus

S. aureus: *Staphylococcus aureus*

S. maltophilia: *Stenotrophomonas maltophilia*

tDN A- PCR: tRNA intergenic length polymorphism PCR

Th: T-helper cell

TNFα: tumour necrosis factor α

TNFs-R: soluble tumour necrosis factor α receptor

tRNA: transfer RNA

Introduction:

Cystic Fibrosis has always been a point of interest in our paediatric pulmonology department. Since the establishment of Cystic Fibrosis (CF) centres in Belgium in 1999 the care for CF patients became better organized. Because the staff was expanded by paramedical co-workers, such as CF nurses, physiotherapists, nutritionists and psychologists, it also became easier to set up both inter-centre and multi-centre studies.

Although we are convinced that peer contacts are psychologically beneficial for patients dealing with CF, we wanted to ensure that our patients did not experience more harm than benefit from these contacts, by patient-to-patient transmission of bacteria.

Pseudomonas aeruginosa is known to be the most important pathogen in CF, and is associated with increased morbidity and reduced life expectancy.

Therefore we set up a first study in the CF rehabilitation centre "Zeepreventorium De Haan" , to compare the genotypes of the *P. aeruginosa* isolates carried by chronically infected patients, during several months (**publication 1,** p. 73).

Because of the ongoing debate of the necessity of cohorting patients, chronically infected by *P. aeruginosa*, we set up a national data bank of *P. aeruginosa* genotypes from these CF patients, in collaboration with all 7 Belgian CF centres (**publication 2**, p. 83).

During the De Haan study we noticed that many of the patients, chronically infected by *P. aeruginosa* also seemed to be infected or even co-colonized with *Achromobacter xylosoxidans (A. xylosoxidans).* Since there is little knowledge about the occurrence and transmissibility of this organism within CF patients, we set up a study to compare genotypes of *A. xylosoxidans* in the same population in the Zeepreventorium (**publication 3,** p. 113).

The clinical significance of this micro-organism is unclear and until now, there is limited evidence for necessity of treatment.

Therefore we set up a retrospective case control study to examine the clinical impact of chronic *A. xylosoxidans* infection (**publication 4**, p. 120).

Chapter I. Etiology of Cystic Fibrosis (CF):

Cystic Fibrosis is a condition caused by a genetic defect that leads to a variety of abnormalities in the CF transmembrane conductance regulator (CFTR).

The CFTR gene is located on the long arm of chromosome 7. The CF gene is large, spans 250 kb, and is composed of 27 exons. As shown in Figure 1 the gene is transcribed into a 6.5-kb messenger RNA that encodes a 1,480 amino acid protein. Since identification of the gene, over 1000 disease-associated mutations in the CF gene have been reported to the CF Genetic Analysis Consortium database (www.genet.sickkids.on.ca/cftr/).

Since it is an autosomal recessive disease, two mutant CFTR genes are necessary to produce a Cystic Fibrosis Phenotype (CFP). This phenotype is characterized by a very wide spectrum, spanning severely ill neonates to newborns with subclinical disease.

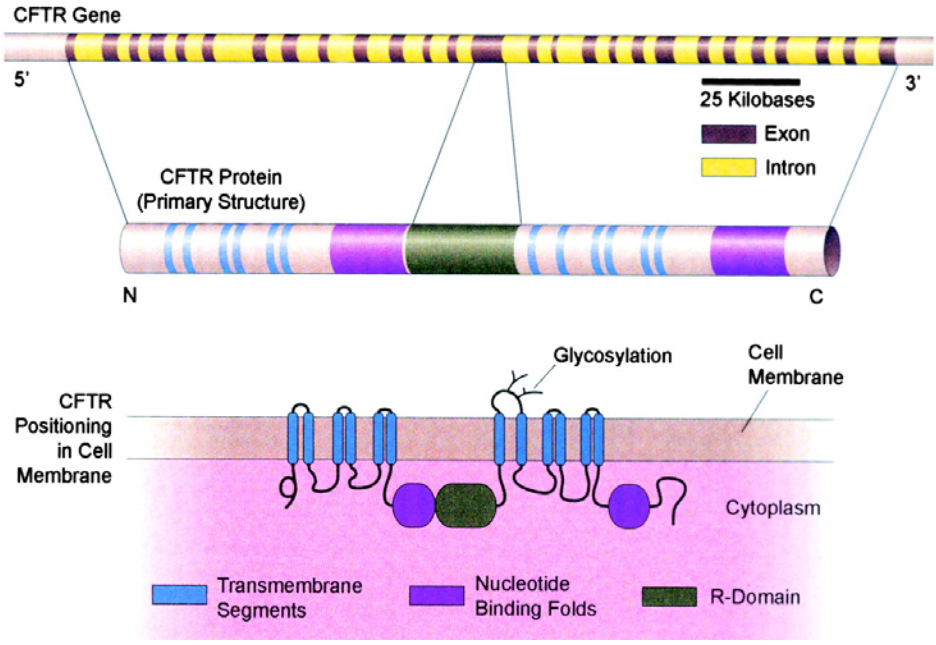

Figure 1. The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) gene and its encoded polypeptide. [1]

The human CFTR gene is located on the long arm of chromosome 7. The mature protein after proper folding, glycosylation, and insertion into the cell membrane is shown at the *bottom of fig.1*. The CFTR protein is a member of the ATP-binding cassette family of transporters. It contains two nucleotide-binding domains that bind and hydrolyze ATP, two dual sets of membrane-spanning segments that form the channel, and a central regulatory (R) domain. The R domain, unique to CFTR, is highly charged with numerous phosphorylation sites for protein kinases A or C [1].

The mutations in the CF gene can disrupt CFTR function in different ways, ranging from complete loss of protein to surface expression with poor chloride conductance. The five major mechanisms by which CFTR function is altered are summarized in Figure 2.

Class I mutations produce premature transcription termination signals resulting in unstable, truncated, or no protein expression.

Class II mutations, usually mis-sense mutations including ΔF508, cause the protein to misfold leading to premature degradation and failure to reach the apical cell membrane.

Class III mutations, primarily located in the two nuclear-binding domains, result in decreased chloride channel activity due to abnormal adenosine triphosphate (ATP) gating.

Class IV mutations are primarily located in the membrane spanning domains that form the chloride channel and demonstrate reduced chloride conductance.

Class V mutations result in reduced amounts of functional protein (rather than no protein production seen in Class I) due to abnormal or alternative splicing.

It is important to recognize that specific mutations may have characteristics of more than one class. Thus, these five mechanisms of CFTR dysfunction are intended to provide a framework for understanding the molecular basis of epithelial cell abnormalities in CF, help predict

observed genotype–phenotype correlations, and develop treatment approaches directed to specific classes of mutations.

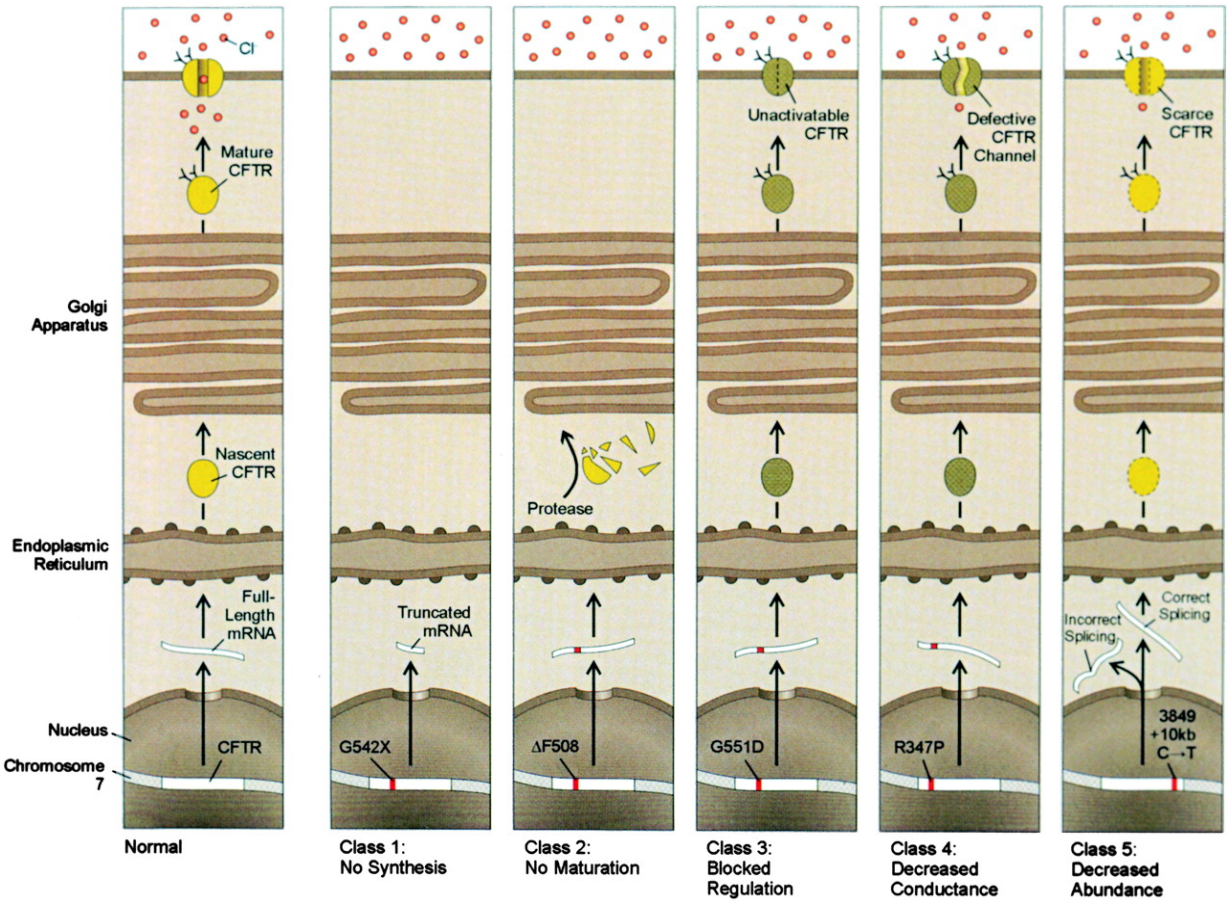

Figure 2. Class I-V mutations of CFTR [1]

CFTR: function and regulation:

After identification of the CF gene in 1989, the 1990s was a decade associated with rapid expansion of knowledge regarding the structure and function of the CF gene product, CF transmembrane conductance regulator (CFTR) protein.

CFTR is an ATP-dependent Cl^- channel that mediates cAMP-mediated Cl^- secretion by epithelia, predominantly those in the pancreas, airways and intestine.

Besides its predominant function as a Cl^- channel it has additional functions:

- There is good evidence that CFTR also regulates amiloride-sensitive epithelial sodium channels [2, 3].
- Possibly the CFTR channel conducts ATP to the extracellular compartment, which serves as an autocrine activator of CFTR itself [4], of the outwardly rectifying Cl^- channels [5, 6], and possibly of other transporters in the apical membrane [7].
- According to Bradbury [8] CFTR plays a role in endocytosis and exocytosis.
- The CFTR protein may also function as a chloride channel in intracellular compartments [9].

A recent review by Mehta [10] focuses on functions of CFTR that do not directly relate to a disease mechanism based on channelopathy. The key concept is that newly synthesized CFTR has to enter lipid vesicles which bud from the endoplasmic reticulum. This is abnormally low in ΔF508 CFTR. Normal wild type vesicular CFTR continuously cycles between exposure at the apical membrane and retrieval in subapical recycling compartment, but this retrieval is abnormally fast in ΔF508 CFTR. The review discusses the relationship between this process and the topic of fat metabolism and the possible links between abnormal fatty acid turnover and inflammatory cascades that are abnormal in cystic fibrosis. AMP-activated kinase, which is bound near the C terminus of the CFTR protein, could possibly integrate some of the abnormalities in lipid metabolism that result from mislocalization of CFTR in clinical disease.

Genotype-phenotype prediction is an important area of CF research. In brief, it has been possible to predict the severity of the CF organ-level phenotype from the genotype with high fidelity with respect to the sweat ducts, the pancreas, and the reproductive system. In contrast,

it is still difficult to identify correlations between genotype and phenotype, *i.e.* severity, of lung disease. For instance, patients who are homozygous for the ΔF508 mutation exhibit a wide spectrum in the rate of development and severity of lung disease.

The failure to generate genotype-phenotype predictions in the lung has led to the notion that both environmental-lung interactions and the genetic background of the host contribute substantially to the severity of CF lung disease. Therefore, a search has been initiated for "modifier genes", *i.e.* genes that modify the effect of CF mutations on lung dysfunction. At present, a number of modifier genes have been identified, based on "candidate selection". Thus far, these genes appear to include those that regulate aspects of innate lung defence and inflammatory cascades [11] (see Chapter III).

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Chapter II. Clinical epidemiology of CF

Epidemiology may be defined as the study of the distribution and of the determinants of disease frequency in human populations.

Clinical epidemiology applies epidemiological principles to a clinical population, i.e. to a population already known to have a particular disease.

a. **Diagnosis**:

Diagnosis of cystic fibrosis is mostly made on clinical grounds (or on neonatal screening, in regions where applied). The gold standard for CF diagnosis is the sweat test, which uses pilocarpine iontophoresis to produce sweat for chloride analysis [12]. According to the US CF Foundation Patient Data Registry Annual Data Report of 1996, 98% of patients have sweat sodium or chloride above 61 meq/L, with a mean sweat chloride of 101.7 meq/l (standard deviation 18.91 meq/l). Seventy percent of the US patients are diagnosed before the age of 1 year, and 90% before their 8th birthday [13]. According to the Belgian CF registry of 2003, 68% of the patients is diagnosed before the age of 1 year, 73% before 2 years and 90% before their 12th anniversary [14].

Late diagnosis is associated with milder disease (better lung function and nutritional status), and lower prevalence of colonization with *P. aeruginosa* .

b. **Life expectancy:**

The median survival age increased significantly during the 2 last decades (Figure 3).

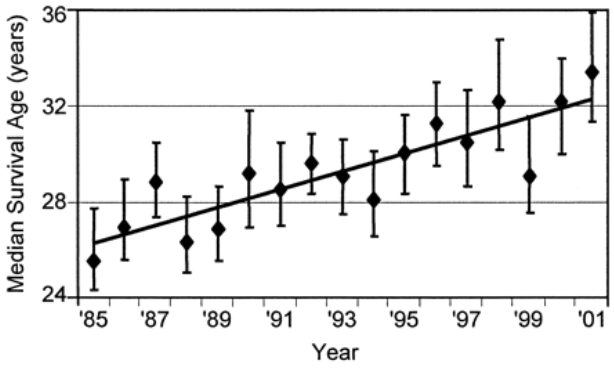

Figure 3. Median survival age in cystic fibrosis, 1985–2001. Data from the U.S. Cystic Fibrosis Foundation Patient Registry showing the age of expected death for 50% of the current Registry population, given the ages of the patients in the Registry and the mortality distribution of deaths for that specific year. The 95% confidence intervals for the survival estimate are denoted by the *vertical bars*. The median estimated survival is 33.4 years for 2001.

c. **Clinical presentation:**

Acute or persistent respiratory symptoms are the most common clinical manifestation (51%), followed by failure to thrive or malnutrition, steatorrhoe or abnormal stools (43%) and meconium ileus or intestinal obstruction (distal intestinal obstruction syndrome: DIOS) (19%).

Congenital bilateral absence of the vas deferens (CBAVD) is a clinical syndrome in which there is a high prevalence of CFTR mutations (14.5% being homozygous for CFTR mutations, 48.1% being heterozygous and 37.5% having no CFTR mutations). Homozygous CBAVD patients frequently have abnormal sweat test, but only a few have clinical symptoms, compatible with CF.

Respiratory symptoms:

Due to progressive lung damage there is a gradual decline in lung function parameters. The mechanisms responsible for this lung damage are described below (see Chapter III).

The organism most frequently reported in CF sputum is *P. aeruginosa.* The prevalence of *P. aeruginosa* colonization varies between countries and between treatment centres in countries. For example, the isolation frequency for *P. aeruginosa* in Canada was 48% in 1995, but varied between treatment centres from 25% to 52%. The reported prevalence in New Zeeland is 62%, it is 62% in French adults and 69% in Ireland [12].

In Belgium the total number of CF patients, registered in the Belgian registry in 2003, was 843, of whom 750 are followed at the 7 CF centres and 280 are considered as colonised by *P. aeruginosa* (37%) [14].

The pathophysiology of infection with *P. aeruginosa* is described below. Other organisms, cultured from the respiratory tract of Belgian CF patients are represented in Table 1.

Results	**n**	**%**
Sterile culture	17	2.2
Normal microflora	146	19.1
Pseudomonas aeruginosa	216	28.2
Mucoïd *Pseudomonas aeruginosa*	116	15.2
Other *Pseudomonas* species	5	0.7
Burkholderia cepacia	5	0.7
Stenotrophomonas maltophilia	32	4.2
Staphylococcus aureus	338	44.2
Haemophilus influenzae	104	13.6

Candida spp.	149	19.5
Aspergillus spp.	118	15.4
Achromobacter xylosoxidans	22	2.9
Other species	120	15.7

Table 1. Organisms cultured from the respiratory tract of Belgian CF patients [14].

Of course, in any discussion of the prevalence of individual CF pathogens age is an important factor (see chapter IV: Microbiology of the CF lung ("early and late" infectors)).

Upper airway problems:

Nasal polyps are found frequently in CF patients, the incidence raising from 10% to 32%. Nasal obstruction is the most common symptom and reason for removal of polyps, but recurrence rate is high.

The sinuses are infected in more than 90% of patients with CF, but bacterial flora does not always correlate with the lower airways. It is however important to consider that sinuses may act as a long term reservoir, and that they are an important risk factor following lung transplantation.

Growth, nutritional status and pancreatic insufficiency:

Until relatively recently many children with CF were of below normal weight and height and had delayed puberty. Those who reached adulthood were of relatively short stature.

Malabsorption due to pancreatic insufficiency leads to deficiencies of the fat-soluble vitamins (vitamin A, D, E and K), some minerals and trace-elements (such as Magnesium and Zinc), essential fatty acids (EFA) and antioxidants (such as vitamin C and E, β-carotene and Selenium).

Factors which contribute to the poor nutritional status of many CF patients include an inadequate energy intake, the often severe and rarely completely controlled intestinal malabsorption and the increased energy demands resulting from chest infection.

Not all CF patients have intestinal problems, about 15% are pancreatic 'sufficient'. However with increasing age, these patients can eventually become pancreatic insufficient, especially those with more 'severe' mutations.

Patients with pancreatic insufficiency tend to have more severe lung disease.

Diabetes mellitus:

Estimates of prevalence of diabetes mellitus vary from 2.5 to 12% of patients, increasing with age.

Liver disease:

About 20% of CF patients have biochemical, echographic or clinical evidence of liver disease, and 5% have overt clinical liver disease, rising from 0.3% in under 5-year-olds to 9% in those older than 16 years. Overt clinical liver disease is commonly associated with serious nutritional problems.

Pancreatitis:

Acute infection/inflammation of the pancreas is almost solely described in pancreatic sufficient patients.

Other endocrine abnormalities:

Insulin-like growth factor 1 is an anabolic hormone and an important marker of nutritional status, liver function and linear growth. A diminished concentration and a correlation with the height score is described in CF patients.

Recent work on leptins, involved in controlling body weight and energy expenditure, showed their beneficial effect on nutritional CF problems.

Fertility problems:

Male reproductive and sexual health:

Infertility is thought to occur in 98% of men with CF, due to the aberrant development of the reproductive portion of the wolffian duct, resulting in absence or atrophy of the vas deferens, vesiculae seminales, ejaculatory duct and epididymis.

The ejaculate is acidic and of low volume. Sexual potency is normal, however severe disease can impair testicular function. Isolated CBAVD can be seen as a 'mild' form of CF.

Female reproductive and sexual health:

Women with CF have anatomically normal reproductive tracts, but abnormalities of cervical mucus have been described. The formation of thick, tenacious cervical mucus may reduce sperm penetration.

Women with severe respiratory disease and poor nutritional status are likely to have amenorrhoea and anovulatory cycles.

With the improved respiratory function, better nutrition and longer survival contemporary young women have higher fertility rates than previous generations.

Osteoporosis:

Osteoporosis is a systemic skeletal disease characterized by low bone mass and micro architectural deterioration of bone tissue, with consequent increase of bone fragility.
The risk factors for developing osteoporosis in CF are: vitamin D and calcium malabsorption, low body weight, decreased physical activity, delayed puberty, male hypogonadism and amenorrhoea, glucocorticoid usage, diabetes, chronic infection with increased cytokines and organ transplantation.

Other organ systems:

Systemic secondary amyloidosis is a complication of long-standing chronic inflammatory diseases due to infection, auto- immune disease and malignancies. Although a high proportion of the patients are chronically infected by *P. aeruginosa*, only around 20-30 cases of amyloidosis are described.
Thyroid gland abnormalities are described, and not only in those patients treated with iodide as expectorant.
Kidney disease such as nephrocalcinosis and nephrolithiasis is sporadically seen.
Secondary damage due to aminoglycoside usage is obvious.

d. **Genetic epidemiology:**

The most common disease-causing mutation is ΔF508 (a deletion on chromosome 7 which results in the loss of a phenylalanine residue at position 508 of the putative protein);
This mutation was first identified on 68% of French speaking Canadian CF patients' chromosomes [15]. Table 2 presents the Belgian data.
The frequency of the ΔF508 mutation differs significantly among various ethnic groups. The highest incidence is reported in Denmark (82% of CF mutations), whereas the lowest

incidence occurs in Algeria (26.3%). In Northern Europe, the overall incidence is approximately 71%, whereas in Southern Europe it varies between 45 and 55% [16].

CF is more common in Caucasian populations, and the incidence varies also within countries. For the UK the incidence is estimated at 1/2400, whereas in Belgium it is 1/3700.
In Israeli Jews (Europe-America) the incidence is 1/3300, in Jews of Asia and Africa 1/9400, in American blacks 1/17000, in the oriental population (Hawai) 1/90000 and in Japan it is limited to 1/320000-680000.

Table 2. Distribution of mutations in the Belgian CF population 2003 [14].

Mutations	n	%
ΔF508	1139	71.4
G542X	49	3.1
N1303K	40	2.5
1717-1G->A	23	1.4
3272-26A->G	18	1.1
R117H	15	0.9
S1251N	14	0.9
2789+5G->A	12	0.8
L927P	12	0.8
W1282X	10	0.6
ΔI507	10	0.6
R553X	9	0.6
3849+10kbC->T	8	0.5
A455E	8	0.5
poly-T tract variations	7	0.4
E60X	7	0.4
394delTT	7	0.4
2183AA->G	6	0.4
Y913C	5	0.3
G970R	5	0.3
W401X	5	0.3
G85E	4	0.3
R334W	4	0.3
Other	85	5.3
Unidentified	94	5.9
Not determined	10	
Total	1624	100.0

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Chapter III. Pathophysiology of respiratory infection in CF

a. Introduction:

The CF gene product, which is the membrane-bound CFTR protein, has been shown to be the chloride-ion channel regulating the transport of chloride ions across fluid-transporting epithelial cells such as exocrine glands. This leads to altered secretions (salty sweats and dehydrated, sticky mucus), blocked ducts and reduced non inflammatory defence of the respiratory tract, which in turn leads to increased inflammatory defence and subsequently to lung damage.

Before the introduction of penicillin, almost all patients died before the age of 5 years, due to *Staphylococcus aureus (S. aureus)* infection.

The most significant CF pathogen during the last 3 decades remains *P. aeruginosa,* which causes most of the morbidity and mortality of these patients.

Below, an overview of the pathophysiology of infections in cystic fibrosis will be given [1].

b. Impact of defective CFTR on airway physiology and mucociliary clearance

The impact of aberrations in transepithelial ion flow on the ionic composition and volume of airway surface liquid (ASL) in CF due to dysfunctional or absent CFTR is an active topic of investigation. ASL consists of two layers above the epithelial surface: a mucus layer and a periciliary liquid layer (PCL) with a height of the extended cilium (~ 7 μm). The PCL volume is tightly regulated to provide a low-viscosity solution for ciliary beat and to lubricate gel-forming mucins secreted from the cell surface. The mucus layer consists of high molecular weight mucins whose properties are altered by water content, ion concentrations, and pH. The mucin gel binds a wide variety of particles for ultimate clearance from the airway (Figure 4).

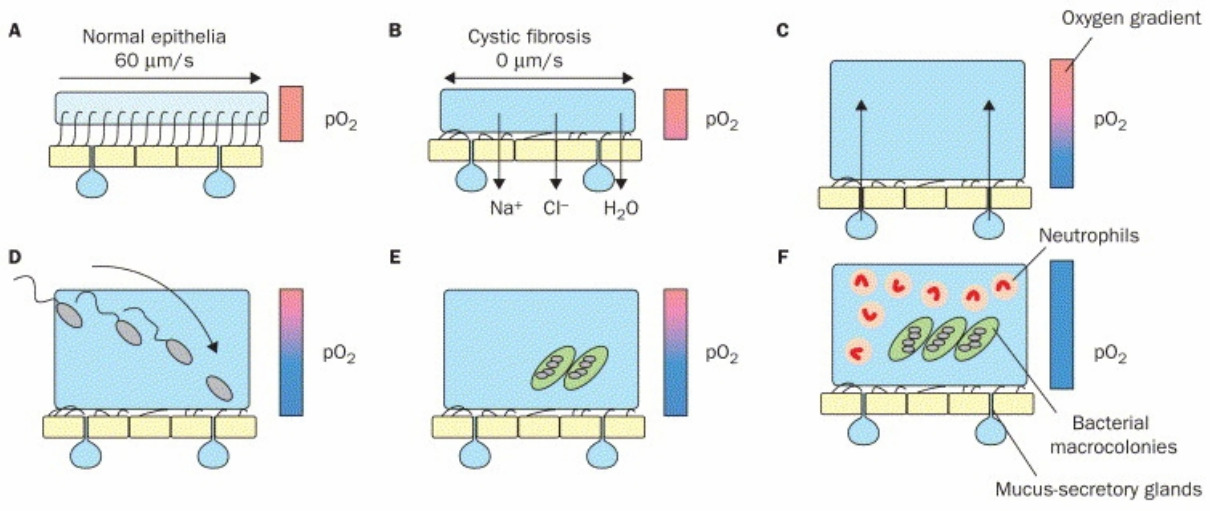

Figure 4. Pathogenic events hypothesized to lead to chronic *P. aeruginosa* colonization.

(*A*) In normal airway epithelia, the presence of a low-viscosity periciliary layer (PCL) of normal volume promotes efficient mucociliary clearance. A normal rate of epithelial cell oxygen consumption (QO_2) results in no gradient in the partial pressure of oxygen (pO_2) within the airway surface liquid (ASL). In the CF airway, (*B*) isotonic volume depletion of the PCL (denoted by *downward arrows* and bent cilia) results in reduced mucociliary transport (*bidirectional horizontal arrow*) and (*C*) persistent mucus hypersecretion (denoted by *upward arrows* from secretory gland/goblet cell units) with time increases the height of the luminal mucus layer/plugs. Elevated CF epithelial QO_2 generates steep hypoxic gradients (*dark colour* in pO_2 *bar*) in the thickened mucus layer. (*D*) *P. aeruginosa* bacteria deposited on mucus surfaces penetrate actively or passively (due to mucus turbulence) into hypoxic zones of the mucus masses. *P. aeruginosa* adapt within the hypoxic environment with increased alginate expression and the formation of microcolonies with potential evolution into biofilms. (*E*) Increased *P. aeruginosa* microcolony density and the presence of neutrophils render the mucus layer more hypoxic. *P. aeruginosa* microcolonies within the hypoxic mucus plugs resist host lung defenses, including neutrophils, and result in chronic airway infection [17].

Two competing hypotheses have been proposed: (*1*) the isotonic "low volume" hypothesis with abnormalities in mucociliary clearance, and (*2*) the "compositional" hypothesis with increased ASL salt concentrations in CF, resulting in inactivation of salt-sensitive antimicrobial peptides [18] (Figure 5).

With regard to the first hypothesis there is no final consensus on the tonicity of ASL in subjects with CF relative to healthy control individuals. Technical limitations of collecting and assaying ASL from the upper and lower airways are a significant obstacle. It is also uncertain whether ASL composition varies along the respiratory tract (i.e., from nasal epithelium to distal airways), in response to chronic inflammation and infection, and within local microenvironments such as submucosal glands or mucus plugs. There is increasing evidence from nasal and bronchial epithelium derived from human and animal sources, that ASL is similar in healthy control individuals and subjects with CF and is isotonic [19-23].

However, using a novel isotopic technique, one investigator suggests that normal ASL concentrations of sodium and chloride are approximately 50 mM and that the ASL concentrations of these ions are elevated to approximately 100 mM in CF [24]. Therefore, the "compositional" hypothesis has not been entirely rejected, especially when considering local microenvironments such as submucosal glands.

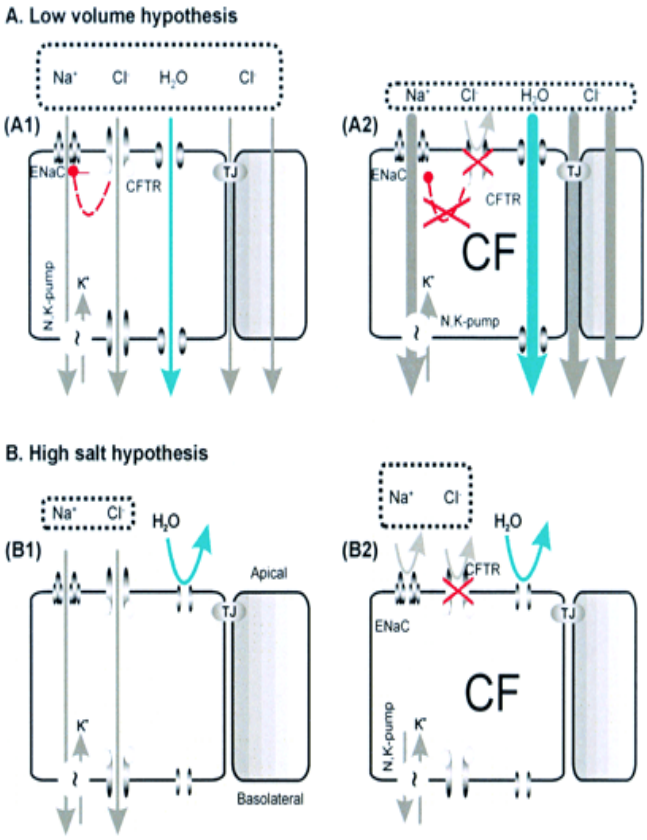

Figure 5. Two hypotheses of how ASL differs in healthy and CF lungs. (*A*) The low-volume hypothesis postulates that normal ASL (*A1*) has salt levels approximately equal to plasma. In CF (*A2*), the removal of CFTRs inhibition of epithelial sodium channels (EnaC) results in abnormally elevated isotonic fluid absorption, which depletes the ASL and leads to reduced mucociliary clearance. Key features of the low-volume model are the parallel pathway for Cl^- via shunt pathway(s) and inhibition of ENaC via CFTR. (*B*) The high-salt hypothesis postulates that normal ASL has low levels of salt as a result of salt absorption in excess of water (*B1*). Even though the epithelium is water permeable, salt is retained in thin surface films by some combination of surface tension impermeant osmolytes. In CF (*B2*), salt is poorly absorbed resulting in excessively salty ASL that inactivates endogenous, salt-sensitive antimicrobial peptides. Key features of the high-salt model are: the lack of an appreciable shunt Cl^- conductance, central importance of CFTRs channel role, no specific role for inhibition of ENaC by CFTR, and a switch from isotonic volume absorption to hypertonic salt absorption as the surface layer thins and traps residual water. [1]

With regard to the second hypothesis, it has recently become more clear that both the accelerated Na^+ absorption and the failure to initiate Cl^- secretion to the abnormal ASL volume plays an important role in the pathophysiology of CF. These abnormalities ultimately lead to depletion of the PCL layer and to the formation of thickened ("concentrated") mucus plaques and plugs adherent to CF airway surfaces [11].

Mucociliary clearance is a primary innate airway defence that, relying to most studies, is reduced in CF. In CF, there is abnormal regulation of the periciliary liquid volume that contributes to reduced mucociliary clearance. Altered viscosity and regulation of submucosal gland secretion may also impair host defence. In addition, the reduced periciliary liquid volume promotes interactions between gel mucins in the mucus layer with cell-surface mucins that hinder clearance of particles from the airways. Clearance of particles from normal peripheral airways by mucociliary clearance can require up to 6 hours, and this can be significantly prolonged in CF airways. Endogenous antimicrobial peptides can suppress bacterial growth for 3 to 6 hours. Thus reduced mucociliary clearance in CF may contribute to

overwhelming of the innate antimicrobial peptides by bacteria and thereby promote the initial endobronchial infection in young children.

c. **Impact of defective CFTR on initial and persistent *P. aeruginosa* infection**

The abnormal composition and mechanical properties of airway secretions does not explain the propensity for the CF airway to become colonized with only a limited number of bacterial pathogens, in particular, *P. aeruginosa*. There are several hypotheses to help us understand that association.

- **Abnormal bacterial adherence to epithelial cells (Prince hypothesis)?**

  Initial infection may be related to increased *P. aeruginosa* adherence to receptors in the CF airway (Figure 4C). CF epithelial cells demonstrate greater adherence of piliated laboratory strains of *P. aeruginosa* compared with control cells, and expression of wild-type CFTR in CF cell lines results in reduced *P. aeruginosa* binding [25-27]. The degree of *P. aeruginosa* binding was greater in nasal scrapings from patients homozygous for ΔF508 compared with compound heterozygotes or carriers [28]. The basis for this increased adherence of piliated *P. aeruginosa* to the apical surface of CF epithelial cells is proposed to be secondary to increased asialoganglioside-1 [25, 26, 29, 30]. Asialoganglioside-1 receptors are increased in cells expressing mutant CFTR and in areas of regenerating epithelium that are likely present in the inflamed CF airway [26, 29]. Asialoganglioside-1 however is not a receptor for clinical mucoid isolates without pili or flagella [29], and therefore this host–pathogen interaction may not be relevant to chronic *P. aeruginosa* infection.

- **CFTR: a receptor for *P. aeruginosa* internalisation (Pier hypothesis) ?**

  In contrast with the former hypothesis that there is increased *P. aeruginosa* adherence to receptors in the CF airway, this hypothesis contradicts the former one and

hypothezises that it is wild type CFTR that is a receptor for *P. aeruginosa*, leading to binding to airway epithelium for subsequent phagocytosis and clearance by desquamation [31-33]. Thus, reduced wild type CFTR leads to reduced *P. aeruginosa* binding, resulting in reduced *P. aeruginosa* clearance from the CF airway. This mechanism can initiate endobronchial infection. The complete LPS outer core is proposed to be the *P. aeruginosa* ligand that binds to wild-type CFTR. Nonmucoid clinical isolates of *P. aeruginosa*, but not mucoid isolates, seem to bind to CFTR and are cleared more rapidly from wild-type versus transgenic CF mice and overexpression of CFTR in transgenic mice resulted in increased clearance of *P. aeruginosa* from the lung. *P. aeruginosa* adaptation within the CF airway is associated with modifications to LPS structure: the specific LPS structures required for *P. aeruginosa* binding to CFTR have not been fully elucidated.

Epithelial phagocytosis probably does not play a role in established infection however, as mucoid *P. aeruginosa* and *S. aureus* are observed primarily within endobronchial mucus and not adherent to the epithelium.

There are some arguments to prefer the Pier hypothesis and to refute the Prince theory:

1. Increased adherence is only seen when cells from patients homozygous for ΔF508 allele are used, yet patients with other alleles or compound heterozygotes for mutant ΔF508 alleles are just as severely affected by *P. aeruginosa.*
2. The major bacterial adhesin to asialo GM1 is proposed to be the bacterial pilus, yet the crystal structure of the *P. aeruginosa* strain PAK pilin subunit has been solved recently, and the site previously identified to bind asialo GM1 was not found to be surface-exposed on the pilin subunit.

3. In the Prince theory, bacterial adherence to asialo GM1 is confirmed by commercially purchased rabbit antisera. These, however, contain high titers of antibodies to *P. aeruginosa* and other antigens (for instance bovine serum albumin). Therefore, one must question the conclusions that there are increased levels of expression of asialo GM1 on CF cells, because of the lack of specificity of the antisera to asialo GM1.

- **Innate immunity and persistence of bacterial infections.**

Innate immune responses provide the first line of defense to airway infection together with mucociliary clearance. Submucosal glands, goblet cells of the large airways, Clara cells within the small airways, and epithelial cells secrete proteins and peptides into the ASL that can kill a broad spectrum of bacteria or modulate the host inflammatory response [34]. There is no evidence for a primary defect in the production of these antimicrobial peptides residing in the CF ASL, except for mannose-binding lectin. Decreased concentrations of mannose-binding lectin, observed in individuals with polymorphisms in the mannose-binding lectin gene may contribute to more rapid decline in pulmonary function and poor survival in patients with CF colonized with *P. aeruginosa* and *Burkholderia cepacia*-complex [35, 36].

As explained above, the changed conditions in ASL and PCL can lead to inactivation of the antimicrobial peptides permitting initial bacterial colonization within the CF airway.

- **Acquired immunity.**

There is no evidence for a systemic immunodeficiency in CF to explain the chronic endobronchial infection. CF patients have no increase in the frequency or severity of infections outside of the respiratory tract and they have normal immune responses to standard immunizations. Patients with CF mount a significant humoral response to *P. aeruginosa* antigens, and there are emerging data that serum antibodies directed against whole-cell *P. aeruginosa* lysates or specific *P. aeruginosa* antigens can be the first markers of *P. aeruginosa* infection in young children with CF [37-38]. Patients with chronic *P. aeruginosa* infection demonstrate high concentrations of antibodies directed against multiple *P. aeruginosa* antigens. However, despite this early and sustained immune response to *P. aeruginosa*, the host is generally unable to clear *P. aeruginosa* from the airways.

However, the potential to eradicate non-mucoid *P. aeruginosa*, and to delay transformation to mucoid species, makes ascertainment of the initial *P. aeruginosa* infection one of the highest priorities in clinical CF management. Traditional methods of *P. aeruginosa* identification (relying on microbiology) leave much to be desired, especially in youg children with CF. Thus, *Pseudomonas* serology could have a potential diagnostic value, in these cases of "early" infection.

A recent editorial [39] illustrates the controversy on this subject, by commenting two publications with contrasting results [40, 41]. A German research group [40] states that regular determination of serum antibodies is reasonable in CF patients with negative or intermittent *P. aeruginosa* colonisation, but not in those with a positive *P. aeruginosa* status. Based on their experience that a rise in antibody titers indicates

probable infection, they recommend eradication treatment, even in the absence of positive culture.

This is however in sharp contrast with the findings of a Dutch group [41] that concludes that "although serological tests are sensitive for identification of chronic *P. aeruginosa* infection, the failure of serological tests to detect early colonisation in young patients emphasises the need for continued reliance on cultures".

The reasons for these contrasting results could be many [39]. Both groups used the same commercially available ELISA test system, but used different cut off values. There were design differences (different periods of repeated measurements). To evaluate whether CF patients are *P. aeruginosa*-free, intermittently infected or chronically infected, *P. aeruginosa*-positive culture remains the "gold standard". In the German study, only 42% of patients could expectorate sputum and thus nasopharyngeal swabs were used for the remaining patients. In the Dutch study the results were based on a sputum cultures in 75% of patients. The greatest difficulty in studying young children with CF-that is the problem of culturing lower respiratory tract secretions-will continu to plague these kind of investigations. For these reasons, non-culture based methods such as serological tests and polymerase chain reaction require further research and evaluation.

d. **Establishment of chronic infection**

With slow proliferation of bacterial micro-organisms and biofilm formation, the stage for persistent infection of adherent mucus is set. The growth of biofilms in thickened mucus plaques creates advantages to the bacteria. Migratory neutrophils penetrate difficultly into the

thickened mucus plaques, and the diffusion of antimicrobial activities into the thick mucus plaques is limited. This evasion of secondary defense mechanisms, coupled with the competitive advantages for bacteria in the biofilm growth modus, leads to the scenario of persistent infection. Bacterial growth in densities sufficient to generate biofilms will probably deplete the mucus plaques of virtually all oxygen, rendering the infected material on airway surfaces anaerobic (see Figure 4, above).

The likelihood that CF airway infections reflect an anaerobic mucus/mucopurulent surface infection has broad implications for the therapy of CF infectious lung disease [11]. The sensitivity of many antibiotics is very different when bacteria are grown under aerobic *versus* anaerobic conditions. For instance, it has been shown that the sensitivity of *P. aeruginosa* to macrolides shifted one to two logs to the left under anaerobic conditions compared with aerobic conditions [42].

e. **Role of cytokines and inflammatory mediators in cystic fibrosis**

Airway disease in cystic fibrosis is characterised by chronic infection and an inflammatory response dominated by a neutrophilic infiltrate. There is incomplete understanding of the relationship between the abnormal CFTR gene product and the development of inflammation and progression of lung disease in CF.

The review by Courtney, Ennis and Elborn [43] gives an overview of the role of cytokines and their dysregulation in the pathogenesis of lung disease in CF.

Airway inflammation in CF seems to be associated with increased production of pro-inflammatory cytokines in the lung. Airway epithelial cells, macrophages, and neutrophils are

all capable of producing cytokines. Several studies have found elevated concentrations of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8, and tumour necrosis factor-alpha (TNFα) in the sputum and bronchoalveolar lavage fluid (BALF) of patients with CF. Their synthesis is promoted by the transcription factor nuclear factor-κB (NF-κB), which plays an important role in intracellular signalling for the production of pro-inflammatory cytokines. IL-10, IL-1 receptor antagonist protein (IRAP), and soluble TNFα receptor (TNFsR) are anti-inflammatory cytokines that are relatively down-regulated in CF airway cells. The principle action of IL-10 is to increase the synthesis of I-κB, the inhibitor of NF-κB. Down regulation of IL-10 leads to increased pro-inflammatory cytokines due to less inhibition of NF-κB actions.

The T helper (Th) cell clones into either Th1 (IFN-γ-producing) or Th2 (IL-4-, IL-5-, or IL-10-producing) cells and thus, the outcome of chronic infections has been thought to depend on the differences in the specific Th cell response. Patients with a higher expression of IFN-γ in bronchial biopsies seem to have milder disease.

In patients, with chronic infection, those with higher IFN-γ production had better lung function.

In chronic *P. aeruginosa* lung infection in mice with a pulmonary Th1 response, there is lower mortality, faster clearance of bacteria, and milder lung inflammation in comparison to mice reacting with a Th2 response.

Peripheral blood mononuclear cells from patients stimulated with *P. aeruginosa* antigen demonstrated a Th2-dominated response in CF patients with stable chronic *P. aeruginosa* lung infection as compared to CF patients without chronic *P. aeruginosa* lung infection. The predominance of a Th1 or Th2 response is thought to depend on the type of dendritic cell that is responsible for the priming of the T cells to new antigens.

As mentioned above, the review on CFTR by Mehta [10] also discusses the possible links between abnormal fatty acid turnover and inflammatory cascades that are abnormal in cystic fibrosis.

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Chapter IV. Microbiology of the CF lung

Since chronic infection of the lower airways in CF is the most important cause of death, it is very important to know the different etiologic agents of these acute exacerbations, which will lead by time to chronic infection.

Acute exacerbations of chronic respiratory disease in CF patients were found to be caused by bacteria (63%), bacteria and virus (13%) and virus (6%), whereas no etiologic agent could be detected in 18% of the exacerbations [12].

Diagnosis of respiratory virus infections is currently achieved by detecting of virus antigens using the ELISA technique, or by showing the presence of raised antibody titers against viruses. Nowadays, the polymerase chain reaction (PCR) for detecting viral DNA or RNA is also applied in this diagnostic field. Viruses as influenza A and B, parainfluenza virus 1 and 3, rhinovirus, adenovirus and respiratory syncytial virus (RSV) are known to cause exacerbations in CF patients [12].

CF has a unique set of bacterial pathogens that are frequently acquired in an age-dependent sequence. The pattern of age-specific prevalence as well as overall prevalence of these pathogens in the CF population in the United States is demonstrated in Figure 6 from the Cystic Fibrosis Foundation Patient Registry data [44]. Of the organisms causing infection in CF, only *S. aureus* may be pathogenic in immunocompetent individuals. *P. aeruginosa*, *B. cepacia* complex, nontypeable *H. influenzae*, *S. maltophilia*, and *A. xylosoxidans* are all considered opportunistic pathogens. Other organisms seen in CF, generally non pathogenic in the healthy host, include *Aspergillus* and nontuberculous mycobacteria.

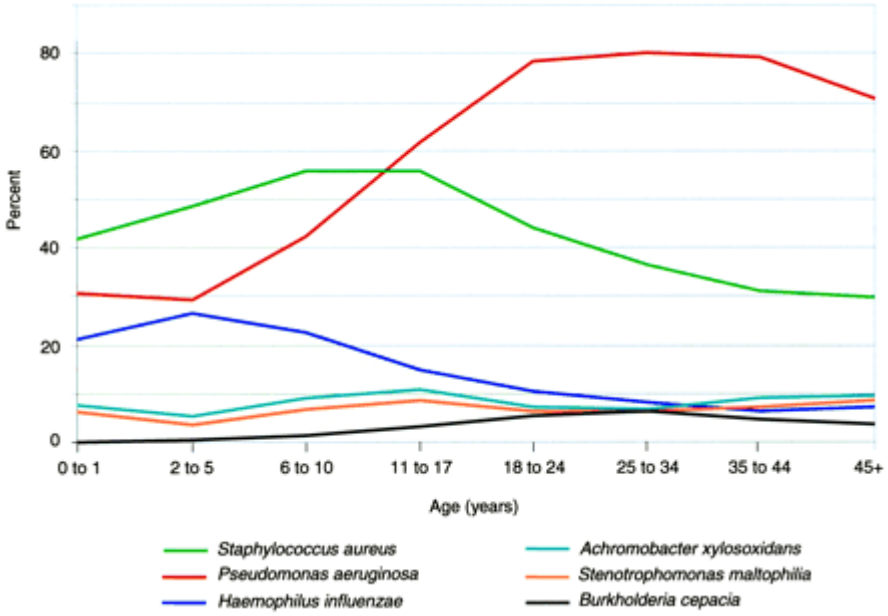

Figure 6. Age-specific prevalence of airway infections in patients with CF. Organisms reported to the U.S. Cystic Fibrosis Patient Registry, 2001. Overall percentage of patients (all ages) who had at least one respiratory tract culture performed in 2001.

We can divide the infecting organisms in '**early infectors'** and **'late infectors'**.

Early infections in CF airways are most frequently caused by *S. aureus* and *H. influenzae*, organisms that may be seen in other young children with chronic respiratory illnesses and in adults with non–CF bronchiectasis. *S. aureus* is often the first organism cultured from the respiratory tract of young children with CF. Whether *S. aureus* is really harmful for CF lungs continues to be debated. Also MRSA (methicilline resistant *Staphylococcus aureus*) is an emerging problem in CF patients.

H. influenzae is also isolated from the respiratory tract early in the course of CF. The *H. influenzae,* infecting patients with CF, is non typeable, thus not prevented by childhood immunization against *H. influenzae* type b. The role of *H. influenzae* in progressive airway

infection and inflammation in patients with CF has not been clearly demonstrated, although it is known to be pathogenic in patients with non–CF bronchiectasis.

P. aeruginosa has been regarded in the previous decades as a 'late infector'. However several recent studies show that infection appears to occur much earlier than believed previously [44, 45, 46]. In a natural history study of patients with CF in the first 3 years of life, the mean age of detection of an antibody response to *P. aeruginosa* was approximately 15 months, whereas the mean ages of first positive upper and lower airway culture were approximately 21 and 23 months, respectively. In total 29 of the 40 patients demonstrated *Pseudomonas aeruginosa* during their first 3 years [45]. In a study of 68 patients with CF, identified by neonatal screening, antibody responses to *P. aeruginosa* were identified, on average, nearly 12 months before positive oropharyngeal (OP) cultures (lower airway bacteriology was not available in these individuals) [46]. Up to 80% of patients with CF are eventually infected with this organism, and acquisition of the organism is associated with clinical deterioration. The source of *P. aeruginosa* isolates in patients with CF has not been clearly established. There is a wide distribution of *P. aeruginosa* genotypes that have been demonstrated in young children [45], suggesting acquisition from environmental reservoirs. Comparison of genotypes from upper and lower airway sources collected simultaneously from patients demonstrates that distinct genetic strains may colonize different anatomic sites in the CF airway [47].

Whereas early isolates phenotypically appear much like environmental isolates, later isolates are more resistant to antibiotics and frequently mucoid. Altough the presence of microcolonies of *P. aeruginosa* was already described in 1980 [48], the existence of biofilms in CF has been fully recognized only recently (see Chapter 3). Biofilms are sessile communities of bacteria on surfaces (Figure 4) with following characteristics: slow growth of organisms, stimulation of production of antibodies that are ineffective in clearing bacteria and inherent

resistance to antibiotics. Therefore it becomes impossible to eradicate biofilm infections even in hosts with intact immune systems.

The presence of *P. aeruginosa* biofilms in infected CF airways was first suggested because of the quorum-sensing signals that the organisms produce to signal cell-density–dependent gene expression. In addition electron microscopy has demonstrated organized clusters and microcolonies of *P. aeruginosa* in expectorated CF sputum consistent with biofilm formation. Subsequently, the presence of local hypoxia within mucus plaques in the airways has been suggested to increase *Pseudomonas* alginate production, which may lead to increased biofilm formation. How biofilm formation develops is described in Chapter 3, Figure 4.
Recently the genome of a laboratory strain of *P. aeruginosa*, PAO1, has been sequenced. *P. aeruginosa* has a very large genome (6.3 Mbp) [49]. This complete genome offers the potential for a tremendous ability to adapt to multiple different environments, including the CF airway. *P. aeruginosa* isolated from CF sputa have even larger genomes than the laboratory strain, PAO1, suggesting that they have acquired new genes during their adaptation, in addition to alterations in those already present [50].

A high frequency of hypermutability has been identified in *P. aeruginosa* isolates from patients with CF. This is likely caused by the specific CF airway with large numbers of infecting organisms and compartmentalization of infection, combined with ineffective host defenses and ongoing antibiotic selective pressure.

True **late infectors** are *B. cepacia*-complex, *S. maltophilia*, *A. xylosoxidans*, nontuberculous mycobacteria and fungi including *Aspergillus*.

B. cepacia is the most feared because of its association with the *B. cepacia* syndrome, which can lead to death due to bacteremia and fatal necrotizing pneumonia. Most patients however

have a more chronic course with decline in lung function and increased mortality. *B. cepacia* is not a single species but rather a group of closely related species, named "genomovars"; thus, the organism should be called *B. cepacia* complex. The vast majority of CF airway infections with *B. cepacia* complex are caused by genomovars II (*Burkholderia multivorans*), III (*Burkholderia cenocepacia*) and V (*Burkholderia vietnamiensis*) [51, 52]. Genomovar III has been shown to spread in epidemics. In this thesis, *B. cepacia* complex was not studied, since the incidence of chronic infection with this organism is low in our centre.

S. maltophilia and *A. xylosoxidans* are encountered more frequently than *B. cepacia* in CF patients with advanced lung disease, but are thought to be less virulent. There is at present limited evidence for a correlation between infection and outcome. Chapter VII of this work will focus on possible patient-to-patient transmission and clinical impact of *A. xylosoxidans.* In a group of 557 CF patients Tan and co-workers [53] reported a prevalence of 2.3% considering only patients with at least three positive cultures over a period of 6 months. In a prospective multi centre German study Steinkamp et al. [54] reported a prevalence of 1.1% among 1419 CF patients.

In the Belgian CF register 2003 [14], gathering 865 patients, a prevalence of 2.9% is mentioned, collecting all patients with at least one positive culture during the year 2003.

These prevalence data can however be disputed, since misidentification of gram negative non-fermenters cultured from CF sputum may occur, because of the diversity of colonial morphologies and biochemical reactivity encountered. For instance, in one study, misidentification of 11% of *A. xylosoxidans* strains was reported [55].

Nontuberculous mycobacteria have been increasingly reported from the respiratory secretions of patients with CF. The species most commonly isolated are *Mycobacterium avium* complex and *Mycobacterium abscessus*. In the US 13% of patients have nontuberculous mycobacteria in their sputum. These patients are older and have a higher frequency of *S. aureus* and a lower frequency of *P. aeruginosa* compared with culture-negative control CF subjects.

Since the CF population is frequently exposed to broad-spectrum antibiotic therapy, fungal colonization of the CF airway late in disease progression occurs frequently. *Candida* spp. are the most frequent colonizers and are usually considered to be harmless commensals. However, *Aspergillus* spp., most frequently *Aspergillus fumigatus*, are isolated from more than 25% of patients [56]. Invasive infections caused by *Aspergillus* are rare in the immunocompetent non transplant CF population, but allergic bronchopulmonary aspergillosis (ABPA) can be a significant problem, wich occurs in about 10 % of CF patients [12]. The cause of ABPA is not an invasive fungal infection but a type I and III hypersensitivity reaction against allergens from *A. fumigatus* in the environment, which leads to a clinical syndrome with wheezing, pulmonary infiltrates and, i untreated, bronchiectasis and fibrosis. Exposure of the airways to high levels of *Aspergillus* allergens and reduced mucociliary clearance in CF, may be a key element in the development of ABPA, especially in atopic patients.

Other molds that have been reported from CF respiratory samples include *Scedosporium apiospermum* and less frequently *Wangiella dermatitidis* and *Penicillium emersonii*. Their clinical significance is unknown.

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Chapter V: Questions to be anwered

As mentioned in the introduction, our concern for patient-to-patient transmission of *P. aeruginosa* amongst the CF patients residing in the CF rehabilitation centre of Zeepreventorium, De Haan, led to a first study (p. 73) in order to answer the following questions:

1. **Do the *P. aeruginosa* colonized patients carry a 'unique' or a 'shared' genotype?** (In other words is it probable that patient-to-patient transmission has occurred in the past?)
2. **Do the patients carry one or more genotypes?**
3. **Do the patients acquire a 'new' genotype during their stay**? (is it possible that patient-to-patient transmission has occurred during the study period?)
4. **Do patients' genotypes correspond with *P. aeruginosa* genotypes found in the rehabilitation centre environment?** (can the patients become infected or co-infected by a *P. aeruginosa* genotype, originating from the environment of the rehabilitation centre?)

The second study (p. 83) tried to set up a national database, in order to answer the following questions:

5. **Do the Belgian *P. aeruginosa* colonized patients carry a 'unique' or a 'shared' genotype?** (is it probable that patient-to-patient transmission has occurred in the past?)
6. **Do the patients carry one or more genotypes?**
7. **Do the patients carry the same genotype, when sampled again, one year later?**

8. **When patients share the same genotype (= cluster type, see later), is there a correlation with the intensity of social contact?**

During the study in the CF rehabilitation centre we noticed that many of the *P. aeruginosa* colonized patients seemed to be infected or even co-colonized with *A. xylosoxidans.* To examine the occurrence and transmissibility of this organism within CF patients, we set up a study to compare genotypes of *A. xylosoxidans* in the same population in the rehabilitation centre. (p. 113).
This study will answer following questions:

9. **Do the patients, chronically infected with *P. aeruginosa* and also co-infected or even co-colonized with *A. xylosoxidans,* carry a 'unique' or a 'shared' genotype of the latter organism?** (is it probable that patient-to-patient transmission has occurred ?)
10. **Do the patients carry one or more genotypes of *A. xylosoxidans*?**
11. **Do the patients acquire a 'new' genotype of *A. xylosoxidans* during their stay**? (is there possible patient-to-patient transmission during the study period?)

The clinical significance of this organism is unclear and until now, there is limited evidence for necessity of treatment. Therefore we set up a retrospective case control study (p. 120) to answer following questions:

12. **What is the prevalence of *A. xylosoxidans* infection (= at least one positive culture) in our CF centre and what is the prevalence of colonization in our CF centre (= at least 3 positive cultures during a period of 9 months)?**
13. **What is the clinical impact of *A. xylosoxidans* colonization?**

Chapter VI. Molecular typing techniques

Methods used for discrimination of genera, species and isolates can be divided into phenotypic and genotypic procedures.

Phenotypic procedures take advantage of biochemical, physiological and morphological phenomena such as cell and colony morphology, cell wall staining properties and the ability of a microbial species to grow under a given set of environmental conditions (e.g. temperature, oxygen dependency, osmolarity and the need for certain nutrients).

Until the early nineties, typing of *P. aeruginosa* for epidemiologic purposes has traditionally relied on bacterial phenotypic characteristics, such as serospecificity of lipopolysaccharides (LPS), susceptibility to bacteriophages and antimicrobial agents, and bacteriocin production and susceptibility.

Although effective in certain clinical settings, some of these methods have been found to be inadequate under conditions in which *P. aeruginosa* undergoes phenotypic conversion (see Chapters III and IV: formation of biofilm, mucoid strains). Furthermore *P. aeruginosa* strains of CF patients are endowed with rough LPS, which renders them refractory to typing with systems that rely on agglutination with antisera or on phage susceptibility. A multicentre comparison of methods for typing strains of CF patients [57] showed that the chromosomal DNA restriction fragment length polymorphism analysis (RFLP) had the greatest discriminatory power, in comparison with 10 phenotypic techniques.

Therefore genotyic procedures were further developed. The starting point of these analyses is that the genome of each individual (and also each germ) is unique. The drawback of these restriction digestion based genotypic typing techniques are the need for a high degree of technical skills, and for a large quantity of high-quality DNA or RNA. Therefore enzymatic amplification of nucleic acid sequences has been applied increasingly for genotyping.

The PCR (Polymerase Chain Reaction) is the prototype nucleic acid amplification method, and it has been extensively evaluated for genotyping [58]. This technique has evolved from a laborious and relatively insensitive assay into an extremely sensitive and highly flexible procedure, since the discovery of thermotolerant DNA polymerases and the development of automated thermal cyclers.

The basis of PCR fingerprinting is the amplification of polymorphic DNA through specific selection of primer annealing sites. Either constant primer sites bridge a single variable sequence domain or primers detect consensus sequences with variable distribution in the DNA. Differences in the distance between primer-binding sites or in the presence of these sites lead to synthesis of amplified DNA fragments which differ in length. These differences can be detected by simple procedures such as gel electrophoresis or chromatography.

Different PCR fingerprinting techniques have been developed and different names have been used for identical techniques. Terms such as amplification fragment length polymorphism (AFLP), arbitrarily primed PCR (AP-PCR), DNA amplification fingerprinting or random amplification of polymorphic DNA (RAPD) are often used indiscriminately and create a 'Tower of Babel' phenomenon. In a letter to the editor in the Journal of Clinical Microbiology in 2003 several CF physicians and microbiologists therefore emphasized the need for harmonization of techniques and technique designations for genotyping clinical isolates of *P. aeruginosa* from CF patients [59]. Epidemiological research, comparable to our studies, has been done in the UK, Canada and Australia [60, 61, 62]. Most of these studies were based on Pulsed-Field Gel Electrophoresis (PFGE).

Since our laboratory had already built up experience to genotype other species with RAPD and fAFLP, we chose to use these techniques [63, 64, 65].

This choice was supported by a publication of Speijer *et al.* [66], who showed that AFLP analysis was the most discriminatory method.
D'Agata *et al*. [67] concluded that AFLP is comparable to PFGE for *P. aeruginosa* isolates.

The culture and genotyping procedure used for *P. aeruginosa* isolates are described in article 1 and 2 (p. 73 and p. 83).

Thus, in these studies, we estimated the genotypic diversity of *P. aeruginosa* colonies, initially by RAPD-analysis, and further with fAFLP-analysis for representative strains of the different RAPD-types observed for each patient. In all cases studied here, isolates with identical RAPD-fingerprints also had identical fAFLP-fingerprints, ensuring that no unrelated isolates were grouped into the same RAPD-genotype. For each patient, all of the RAPD-products were always obtained during the same thermal cycling and electrophoresis run, to avoid differences due to the limited reproducibility of the technique. fAFLP is generally known to be more reproducible and - due to automated digitisation of the fingerprints – it makes possible large-scale comparison of hundreds of fingerprints, an endeavor that is impossible with RAPD-analysis.
This combined approach of a rapid and cheap initial screening technique (RAPD-analysis) and a more sophisticated, more reproducible and digitized, but also more expensive and laborious technique (fAFLP-analysis), enabled us to genotype a large number of isolates in an affordable, reasonably convenient and a highly reliable and discriminatory manner. Moreover, the established library of fAFLP-fingerprints of CF *P. aeruginosa* strains can be used for further comparisons and long-term studies.
For *A. xylosoxidans* (publication 3, p. 113) the same molecular methods were used, after identification of the organism, as described above.

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Chapter VII. To segregate or not to segregate, that's the question…!

a. Introduction: literature data about transmissibility of pathogens other than *P. aeruginosa* and *A. xylosoxidans*.

Since the early nineties, patient-to-patient transmission has been debated extensively. Saiman [69] discussed transmission of the several potential pathogens such as *B. cepacia complex, P. aeruginosa*, MRSA, *S. maltophilia, A. xylosoxidans*, nontuberculous mycobacteria and viruses. In 2004 a review of the same author updated the knowledge about transmission [70].

For ***B. Cepacia complex*** studies from the USA, Scotland, England and Canada demonstrated transmission associated with close contact in social settings. These studies were performed by aid of accurate identification and molecular typing, provided by reference laboratories (i.e. the microbiology laboratory in Ghent, under the surveillance of Peter Van Damme). This knowledge led to disbanding of CF summer camps worldwide [71, 72].

Also transmission in health care settings has been documented, in association with hospitalisation, poor adherence to handwashing, contaminated respiratory therapy equipment and possibly contaminated hospital showers. Health care workers (HCW) do not seem to be a reservoir for *B. cepacia* complex strains (during a 3-month study 73 throat cultures from seven HCW remained negative [73]).

While direct, indirect and droplet spread are demonstrated, true airborne transmission seems less likely.

Some genomovars (as *B. cenocepacia*) are more likely to be spread from patient to patient and to be associated with epidemic outbreaks. *B. cenocepacia* is also known to replace less harmful members of the *B. cepacia* complex, in colonized patients.

Numerous infection control measures that were successful in preventing transmission of *B. cepacia* in CF patients have been described, such as education, intervention in non-health care and health care setting, environmental decontamination and improvement of laboratory practises [70].

In summary, *Burkholderia* species can be transmitted from one patient to another. Thus, CF patients infected with *B. cepacia* complex must avoid close contact with other CF patients, including those already harbouring *B. cepacia* complex strains, to avoid acquiring potentially more virulent strains.

S. aureus is often the first pathogen to infect the respiratory tract of CF patients. In the pre-antibiotic era, the organism caused substantial morbidity and mortality in young children, however with effective antibiotic treatment, the burden of *S. aureus* colonization seems less threatening than of chronic infection with *P. aeruginosa.*

The infection with *S. aureus* usually originates from the anterior nares. The same genotype in nose and subsequently in the lower airways has been demonstrated in non CF [74, 75] and CF patients [76]. In the latter study patients without recent antibiotic treatment had a higher prevalence of nasal colonization, than those, treated recently or than healthy controls. *Staphylococcus aureus* easily spreads in families with or without CF, but loss or replacement by another strain is frequently seen in family members. In CF patients however infection or colonization with the same strain has been documented (for at least 1 to 2 years) [77]. There is a significant increase of methicillin-resistant *S. aureus* (MRSA) in the CF population. In 2001

7% of registered American CF patients harboured MRSA. The clinical impact of MRSA in CF remains uncertain.

One study showed that colonization is frequently brief [78], but another showed persistence of the same clone in a CF patient for several years [79]. Transmission from patient-to-patient can occur (also from non CF patients), thus preventive measures must be taken in in- and outpatient facilities.

In the literature increasing prevalence of ***S. maltophilia*** has been reported over the last decade.In our CF centre the prevalence of *S. maltophilia* positive cultures was 14.3% in 2003, but only 2.3% of patients could be considered as chronically infected (at least 3 positive cultures during 6 consecutive months).

Marchac *et al*. [80] reported a raising prevalence from 3.3% to 15% over a period of 10 years ('91-'99).

Goss *et al*. [81] reported a prevalence of 8.7% for the period of 1991-1997, based on data of the US CF Registry. Talmaciu and co-workers [82] reported a prevalence of 19% from 1993 to 1997.

These papers tried to investigate the possible morbidity of this pathogen. Talmaciu and Marchac compared patients with at least one positive sputum culture with age-matched CF patients who had never been infected by *S. maltophilia.* Higher use of antibiotics seemed to be a risk factor in acquisition.

Marchac could not show a deterioration in lung function, for the 2 years after acquisition.

Goss could not show a difference in survival rate, during a 4 years follow-up study.

Denton *et al.* [83] showed that this organism is widespread. The homes of both colonized (26% positive) and noncolonized (42% positive) CF patients, the hospital wards (32% positive) and the CF Clinic (17% positive) were contamined by *Stenotrophomonas maltophilia.* They also showed that clinics may even harbour the same clone for a year.

Only a few studies have tried to define the transmissibility between patients, based on PFGE or PCR-techniques.

Valdezate *et al.* [84] and Vu-Thien *et al.* [85] showed that most isolates were unique, but could persistent for a long time in an individual patient.

Krzewinski *et al.* [86] demonstrated that in 3 of 6 US CF centres, two patients at each were infected by the same clone, although 2 pairs did not have an epidemiologic link.The Spanish study by Valdezate *et al.* [85] showed 3 patients carrying the same strain.

Very few studies [87, 88] have used molecular typing techniques to examine the possibility of patient-to-patient transmission of **Nontuberculous Mycobacteria**. These studies could not demonstrate shared strains.

Patient-to-patient transmission has not been studied yet for **fungi and molds**, probably because it is almost certain that they are acquired from the environment, because they are ubiquitous (and thus unavoidable) in nature.

Respiratory viruses are important pathogens in CF patients. These patients do not seem to be more susceptible to respiratory viral infections than their siblings or age matched controls [89, 90] but the clinical course of the viral infection can be more severe (especially RSV, Influenza A and adenovirus infections). Transmission is obligatory from other infected persons, via direct, indirect and droplet infection. Prevention therefore is very difficult. Prophylactic strategies as administration of monoclonal antibodies against RSV (Paluvizumab) are on trial.

Vaccination with an Influenza vaccine is recommended for CF patients, 6 months of age and older, and their close contact persons.

b. Transmissibility of *Pseudomonas aeruginosa*

Numerous studies have attempted to identify the initial source of *P. aeruginosa* in CF patients, but this remains unknown for most patients. *P. aeruginosa* has been shown to survive for prolonged periods; nonmucoid strains suspended in saline can survive on dry surfaces for 24 h, mucoid strains can survive 48 h or more, and strains suspended in sputum of CF patients can survive on dry surfaces for as long as 8 days. Therefore it seems logical that patient-to-patient transmission can occur.

Potential sources of *P. aeruginosa*

P. aeruginosa is widespread in the environment [91]. The organism has been isolated from several in- and outpatient sources, such as sinks and tap water in paediatric CF wards [92, 93], childrens' toys and soap [94] and lung function equipment [95]. In some articles these environmental strains are compared genotypically with the patients strains. Occasionally, matches are found, but it always stays unclear whether the patients are the initial source of environmental contamination or vice versa [92, 96]. The study by McCallum *et al.* [97] however showed that a strain carried by 4 adults patients of the same CF centre could not be found from the environment (sinks, drains, toilets, showers, communal surfaces), although repeatedly cultured.

The home nebulizer was also studied as a source of cross- infection: 25 to 50% of the studied nebulizers carried *P. aeruginosa* [98, 99]. However, with the implementation of vigorous cleaning and disinfection (as recommended and taught by the CF centres), this high percentage of positive culture is expected to decrease. A recent Belgian study [100] evaluated the efficacy of 5 methods of disinfection of the mouthpieces and facemasks of nebulizers,

indicating the concerne that exists among CF caregivers, regarding cross-infection from nebulisers.

Other, more rare environmental sources of contamination are described: whirlpools, hot tubs, swimming pools or dental equipment. However, standard chlorinated swimming pools and dental equipment that is cleaned, disinfected and sterilized according to standard procedures do not harbour *P. aeruginosa.*

The organism can also be cultured from the hands of CF patients and HCW and studies even demonstrated a correlation between contaminated sink drains and hands, washed in these sinks [92, 101]. Droplet infection has been demonstrated by isolation from agar plates, placed 1.5 to 3 feet (= ± 45 to 90 cm) from a coughing CF patient [92, 94]. Data about true airborne transmission, based on cultures of air samples, are controversial [94, 95, 102]. Until recently, it was believed to be unlikely that *P. aeruginosa,* from the sputum of a CF patient, could remain suspended in the air long enough to enable transmission. The recent study by Jones *et al.* [103] however showed that epidemic *P. aeruginosa* strains were isolated from room air where patients performed lung function tests, nebulisation and airway clearance, and not from the inanimate environment. They concluded that aerosol dissemination may be the most important factor in patient-to-patient spread of epidemic strains.

Patient-to-patient transmission

Over the last 2 decades, worldwide, investigators tried to find evidence for patient-to-patient transmission.

Early studies in CF siblings first demonstrated shared strains [94, 95, 104]. These studies were based on phenotypic methods.

In the 1980s, the discovery of an epidemic, multidrug resistant strain of *P. aeruginosa* (confirmed by serotyping and pyocine typing and not yet by molecular techniques) in the Danish centre led to implementation of several infection control measures, such as a separate clinic for *P. aeruginosa* colonized patients and better hand hygiene for both patients and HCW. This led to a decreased incidence and prevalence of *P. aeruginosa* infection [105, 106, 107].

Genotyping techniques such as macro-restriction analysis in combination with pulsed-field gel electrophoresis, randomly amplified polymorphic DNA-analysis (RAPD) and amplification fragment length polymorphism (AFLP) have enabled reasonably accurate determination of the clonal relationship of *P. aeruginosa* isolates from patients within a CF centre or region [57, 66, 108, 109]. Nevertheless, different conclusions on the transmissibility of this pathogen have been drawn from two recent molecular epidemiological studies from two large centres - without segregation policy - in Canada [61] and in Australia [62].
The Australian cross-sectional study found a widespread clone of *P. aeruginosa* in 55% of the 118 infected patients in a paediatric CF clinic. In contrast, the Canadian longitudinal study - over two decades – showed a low risk of patient-to-patient spread among 174 patients. Cross-sectional and longitudinal studies in Liverpool [97, 110], Manchester [103, 111, 112] and Sheffield [113] however have provided compelling evidence for transmission of highly transmissible strains.

This raised once more the issue of the risk of cross-infection associated with CF holiday camps. Oyeniyi *et al.* [114] has shown that the five *P. aeruginosa*-negative patients who attended a winter camp in Spain together with 17 patients who were already colonized with *P. aeruginosa*, all acquired *P. aeruginosa* strains that were identical to strains carried by the colonized patients. Hoogkamp–Korstanje *et al.* [115] however reported an incidence of cross-

infection of 7% in previously *P. aeruginosa*- negative individuals (91 CF-patients who attended a CF-camp had respiratory cultures performed on arrival, after two weeks, after two months, and regularly thereafter). The authors concluded that the overall risk of acquisition was comparable to that occurring in the community, and that it was trivial compared with the obvious joy and social benefit derived from a holiday camp.

A Brazilian study [116] in an outpatient CF-clinic also concluded that the risk of cross-infection is low.

In the US, the time to acquisition of *P. aeruginosa* was shorter in infants diagnosed by neonatal screening than in those diagnosed by symptoms [117-120]. This was attributed to crowded clinic conditions, early use of aerosol equipment and early exposition to 'centre care', thus to patient-to-patient transmission.

In summary, it has been demonstrated that CF patients can carry the same strain of *P. aeruginosa.*

There is more evidence for patient-to-patient transmission, than for acquisition of an epidemic strain from the environment. Patient-to-patient transmission is generally associated with close and prolonged social contact.

i. Article 1: ***Epidemiology of Pseudomonas aeruginosa in a cystic fibrosis rehabilitation centre.***

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Epidemiology of *Pseudomonas aeruginosa* in a cystic fibrosis rehabilitation centre

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ABSTRACT: ***Pseudomonas aeruginosa*** **is the leading pathogen in cystic fibrosis (CF) lungs. Since there is great concern about clonal spread in CF centres, this study examined the *P. aeruginosa* genotypes of colonised residents of a CF rehabilitation centre.**

The isolates from the sputum of 76 *P. aeruginosa*-colonised patients were genotyped by fluorescent amplified fragment length polymorphism on arrival and departure.

A total of 71 different *P. aeruginosa* genotypes were identified from 749 isolates. Forty-nine patients had one genotype, 20 had two genotypes and seven had three. Forty-four patients had one or more genotypes in common with other patients (*i.e.* cluster types). Thirty-two patients were colonised by a single genotype not shared by any other patient. Thirty-eight of the 44 patients with a cluster type already carried their cluster type strain(s) on arrival. Patient-to-patient transmission could not be excluded for eight patients. For five of these, this infection was transient. None of the environmental *P. aeruginosa* isolates had a genotype similar to the patients' genotypes.

In summary, most patients were colonised by only one or two *P. aeruginosa* genotypes and the risk of persistent patient-to-patient transmission was low during the study period (4%). Most patients with a cluster-type strain carried this strain on arrival, indicating that transmission could have happened in the past. No environmental contamination could be established.

KEYWORDS: **Cystic fibrosis, epidemiology, *Pseudomonas aeruginosa***

Pseudomonas aeruginosa has been the leading pathogen in cystic fibrosis (CF) lung pathology over the last three decades [1–3]. After initial infection, colonisation (as defined by the criteria of DÖRING *et al*. [4]) leads to the destruction of lung tissue and reduction of lung function, which may result in early death. The US CF Foundation database reported that, in 1996, the median survival of CF patients who were colonised with *P. aeruginosa* was 28 yrs, while the median survival for noncolonised patients was 39 yrs [5]. Loss of lung function has been clearly demonstrated by KEREM *et al*. [6], who showed that patients who were colonised with *P. aeruginosa* at the age of 7 yrs had a mean forced expiratory volume in one second (FEV_1) that was 10% lower than that of noncolonised patients. Although the emergence of a mucoid colonial morphotype is a more unfavourable prognostic factor than the presence of nonmucoid *P. aeruginosa* [7], the latter forms an important microbial reservoir from which mucoidy, bacterial biofilms and chronic colonisation are established [8].

Prevention of chronic *P. aeruginosa* colonisation by appropriate antibiotic therapy is now common practice once a ''new'' infection by the organism has been identified [9, 10]. Currently, spread of highly transmissible strains in some CF centres has caused great concern, particularly when such strains are multi-resistant and responsible for primary infection [11–16]. Other studies, however, have failed to find evidence of clonal spread [17–20].

In Belgium, many patients are referred to the CF rehabilitation centre ''Zeepreventorium'' in De Haan, for either a short or prolonged stay, in order to learn specific physiotherapeutic techniques, such as autogenic drainage. This situation has led to justifiable concern among patients and physicians about the risk of cross-infection. Therefore, during 2001 and 2002, *P. aeruginosa* isolates from 76 patients, together with environmental isolates, were genotyped to investigate the risk of patient-to-patient transmission.

METHODS

Patients

All 76 *P. aeruginosa*-colonised patients who attended the rehabilitation centre from January 8 to April 30, 2001, and from September 1, 2001, to October 31, 2002, with a total duration of stay of 8,218 days (median 63 days), were enrolled in

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this study. Patients were aged 5–38 yrs (mean 20.5 yrs) and 33 were male (table 1). Seven patients stayed in the centre during the first period, 42 during the second period and 27 during both periods. Patients were housed in separate rooms, but dining and living facilities were shared. Patients infected with *Burkholderia cepacia* complex are not admitted to the rehabilitation centre. Patients were mostly referred from one of the seven Belgian CF centres, but also some were from German and French centres.

From 1992, infection control measures were instigated to reduce cross-infection from the environment, *i.e.* decontamination of the sinks in each patient room, patient segregation during physiotherapy according to *P. aeruginosa* colonisation and exclusion of patients harbouring *B. cepacia* complex from the centre. Nevertheless, *P. aeruginosa*-colonised patients continued to share the same physiotherapy room, each patient using his/her own nebuliser and physiotherapeutic devices, which were decontaminated separately after each session.

Sampling

Sputum samples were taken from all patients at least on arrival and departure, and were collected at the end of a physiotherapeutic session to ensure that the samples originated from the deeper airways. Environmental samples (10 mL) were taken during the study period from sink drains of the bedrooms and the recreation rooms. Some patients were sampled >20 times during the study period.

Microbiology

Sputum samples were inoculated onto McConkey agar (BBL Becton Dickinson, Cockeysville, MD, USA). After 2 days of incubation at 37℃, different-looking lactose-negative colonies were picked, subcultured on 5% sheep blood agar (BBL Becton Dickinson) and tested for oxidase. Only oxidase-positive colonies were further investigated, using tRNA-PCR [21].

Genotyping

For each patient, all *P. aeruginosa* isolates exhibiting different colonial morphology on McConkey agar were genotyped by arbitrarily primed PCR, using alkaline cell lysis for DNA extraction [22], and randomly amplified polymorphic DNA analysis (RAPD) Ready-to-Go beads (Amersham Biosciences AB, Uppsala, Sweden) and primer ERIC2 (AAGTAAGTGACTGGGGTGAGCG) at an annealing temperature of 35℃, as described previously [23]. This enabled the number of isolates that were subsequently genotyped by the more laborious fluorescent amplified fragment length polymorphism (fAFLP) method to be reduced, since only single representatives of each RAPD type were genotyped using this procedure.

Total bacterial DNA was isolated from fresh cultures on Tryptic Soy Agar using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). fAFLP was carried out as described below. A combined restriction-ligation procedure was used, in which 10 ng of total genomic DNA was incubated with 2 pmol of EcoRI adapter, 20 pmol of MseI adapter, 1 U of EcoRI (Amersham Biosciences), 1 U of MseI (New England Biolabs, Beverly, MA, USA), 50 mM NaCl, 50 ng bovine serum albumin per μL (Roche, Basel, Switzerland) and 4 U of T4 DNA ligase (Amersham Biosciences), in a total volume of 10 μL of 1× reaction buffer for 3 h at 37℃, after which the mixture was diluted 20 times with Tris-buffer (Tris 10 mM, EDTA 0.1 mM, pH 8.0). For the selective amplification of the restriction fragments, 5 μL of the diluted restriction-ligation mixture was used for amplification in a volume of 10 μL under the following conditions: 0.4 μM 6-tetrachlorofluorescein-labelled EcoRI+0 primer, 1.2 μM MseI+C primer (E1; Eurogentec, Seraing, Belgium), 0.2 mM deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1× reaction buffer and 1 U of GoldStar™ DNA polymerase (Eurogentec). After 2 min of incubation at 72℃ and at 94℃, the cycling conditions were 36 cycles of 30 s at 94℃, 30 s at 65–56℃ and 60 s at 72℃. During the first 13 cycles, the annealing temperature was lowered by 0.7℃ per cycle. After an additional 10-min incubation at 72℃, the samples were cooled. An overview of PCR primers and adapter sequences is shown in table 2.

A combination of 12 μL deionised formamide, and 0.3 μL GS-400 high-density size standard and 0.2 μL GS-500 size standard (which both contain ROX-labelled fragments in the range of 50–500 bp) were added to each 1 μL of PCR product. This mixture was then electrophoresed on an ABI PRISM 310 (Applied Biosystems, Foster City, CA, USA).

A similarity matrix was calculated using the BaseHopper programme [21], and from this a similarity tree was constructed by neighbour joining, using the programme PHYLIP [24]. Fingerprints that were clustered to >90% similarity were visually checked to enable final decisions with regard to similarity. Visual checking of fingerprints that were assessed by the software as having <90% similarity showed that such fingerprints always differed from each other by at least three major peaks. Visual interpretation of fingerprints assessed by the software as having ~90% similarity led, in some cases, to the conclusion that the fingerprint was identical. Therefore, a final decision with regard to clonality of isolates possessing fingerprints with ⩾90% similarity, according to the software, was based on human interpretation.

Statistical analysis

Data are presented as mean±SD or median (interquartile range or SEM). Statistical analysis of the epidemiological data was carried out using the unpaired t-test when groups were normally distributed and the Mann-Whitney rank-sum test when the normality test failed. A 95% confidence interval for the difference in median time in the centre between the group with a possible patient-to-patient transmission and the group without transmission was obtained using a bootstrapping procedure [25].

RESULTS

A total of 749 *P. aeruginosa* isolates from 76 patients for which the colony morphology on McConkey agar was different were genotyped by arbitrarily primed PCR (RAPD). For each patient, at least one representative of each different RAPD type was further genotyped by fAFLP, enabling digital comparison of the genomic fingerprints. Figures 1 and 2 represent some of the fAFLP-fingerprints obtained, with figure 2 representing details of three different fAFLP fingerprints in a superimposed manner.

Only 71 different *P. aeruginosa* genotypes were found among the 749 isolates, indicating that, in individual patients, isolates

TABLE 1 Clinical characteristics of the patients

Patient No.	Age yrs	Sex	Colonised since	Stay duration days (periods)	FVC %	FEV_1 %	Cluster type
1	15	M	1992	14	127	137	Z and Y
2	17	F	1984	18	100	63	Z
3	15	F	1996	144	107	100	Y
4	15	M	1991	48 (2)	95	89	Y
5	19	F	1998	89 (2)	134	112	Y
6	13	F	1991	236 (2)	86	76	Y
7	26	M	1992	98 (2)	41	25	Y
8	21	F	1996	25	77	50	Y
9	5	F	NA	38	NT	NT	Y
10	25	M	<2001	178 (4)	69	32	Y and R
11	7	F	2000	115	45	38	Y
12	10	F	<1999	68 (3)	73	51	X
13	16	F	<2000	84 (3)	69	51	X
14	18	F	<1994	253 (3)	83	65	W
15	8	F	NA	86 (3)	105	83	W
16	21	M	<1999	430 (3)	52	23	U
17	26	F	<1999	213 (4)	48	18	U
18	17	F	<1997	39 (3)	70	37	U
19	26	M	<1997	78 (2)	107	82	U and P
20	31	M	1998	31	NA	NA	U
21	19	M	1992	84	46	31	T
22	8	M	<2001	160 (6)	NT	NT	T
23	9	M	1996	391 (7)	NA	NA	T
24	15	F	1991	25	88	48	T
25	30	M	<1991	41 (2)	53	22	R
26	30	M	<1998	276	48	16	Q
27	18	F	1993	94 (4)	72	48	Q and M
28	16	F	1994	39 (2)	102	100	Q
29	13	M	1996	56 (2)	92	95	P and O
30	27	M	<2000	65 (2)	62	36	P
31	27	M	<2000	122 (3)	35	14	P
32	21	F	<2000	41 (2)	55	41	P and M
33	14	F	NA	102 (2)	116	99	P and O
34	22	F	1985	35	34	22	O
35	26	F	1983	79 (2)	74	47	O and M
36	23	F	<1999	11	103	47	O
37	18	M	<1999	104 (4)	96	85	O
38	18	F	<2000	546 (3)	83	60	O
39	16	M	<1999	211 (2)	71	54	J
40	13	F	<1999	285 (5)	62	46	J
41	33	M	<1990	44	44	22	M
42	28	F	1977	38	63	29	M
43	20	F	<2001	45 (2)	73	76	K
44	15	F	<2001	42 (2)	100	84	K
45	22	F	1991	49	49	25	
46	18	F	<2000	89 (4)	76	49	
47	34	F	<1999	59 (2)	65	34	
48	20	F	<2000	37	40	25	
49	16	F	<1993	321 (3)	52	36	
50	22	M	<1996	37 (2)	62	35	
51	37	F	<1989	27	89	56	
52	22	F	1995	44	104	85	
53	32	M	1993	456	47	28	
54	20	M	<1999	158 (2)	51	35	
55	33	M	1984	28	83	57	
56	12	F	1989	35	67	48	
57	27	F	1993	62 (2)	67	48	
58	15	M	1993	25	82	70	
59	29	F	NA	59	56	22	
60	21	M	1998	36	32	20	
61	26	M	1993	84 (2)	30	21	
62	22	M	NA	31	43	21	
63	28	M	<1990	81 (2)	47	23	
64	26	M	<2000	111 (2)	46	21	
65	23	F	1990	21	96	75	
66	22	M	1992	56 (2)	93	48	
67	10	M	<2000	85 (4)	126	115	
68	12	F	NA	28	65	48	
69	34	F	1985	49 (2)	85	62	
70	26	M	<1988	35	27	17	
71	26	F	1984	59 (2)	42	24	
72	14	M	<2000	79 (2)	100	27	
73	5	M	NA	204 (2)	64	65	
74	18	F	NA	23	103	96	
75	23	F	1991	64 (2)	53	25	
76	19	F	1982	17	46	49	

FVC: forced vital capacity; FEV_1: forced expiratory volume in one second; M: male; F: female; NA: data not available; NT: not tested because of young age or mental retardation.

with different colonial morphology mostly belonged to the same genotype. Fifty-seven of these genotypes were only found in a single patient (distinct genotypes), while 14 were found in more than one patient (cluster genotypes). More than half of the patients (49) carried only one genotype, 20 carried two genotypes and seven carried three genotypes.

Thirty-two patients (42%) were colonised by one or more strains with distinct genotypes, 32 patients (42%) had one or more genotypes belonging to at least one cluster, and 12 patients (16%) carried both distinct and cluster strains.

Of the 44 patients with cluster strains, 36 carried strains that belonged to only one cluster and eight had strains belonging to two clusters. There was a statistical difference between the age

TABLE 2 Adapter and primer sequences used for fluorescent amplified fragment length polymorphism-based genotyping

Adapters and primers	Sequence
EcoRI adapter1	5′ CTCGTAGACTGCGTACC
EcoRI adapter2	5′ AATTGGTACGCAGTCTAC
MseI adapter1	5′ GACGATGAGTCCTGAG
MseI adapter1	5′ TACTCAGGACTCATC
EcoRI+0 primer	5′ (tet)GACTGCGTACCAATTC
MseI+C primer	5′ GATGAGTCCTGAGTAAC

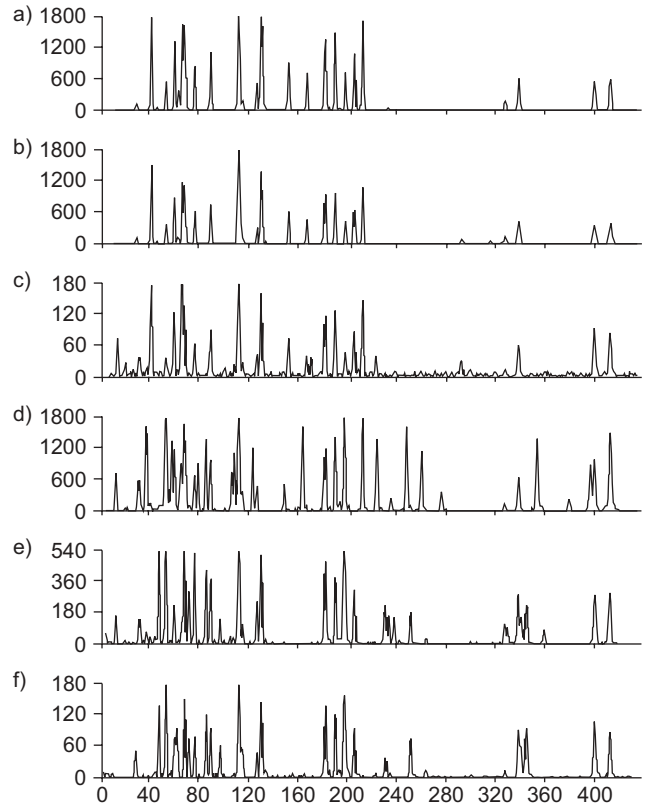

FIGURE 1. Fluorescent amplified fragment length polymorphism fingerprints of *Pseudomonas aeruginosa* isolates from different patients, illustrating different genotypes: a–c) three different patients belonging to cluster Y; d) one patient belonging to cluster U; e, f) two siblings belonging to cluster J.

of the patients with cluster strains (19±6.95 yrs (1.05)) *versus* the age of patients with distinct strains (22.3±7.49 yrs (1.32), p=0.04). The patients with a cluster strain had similar lung function test results when compared with those with distinct strains (FEV1: 49.0% (31.5–82.5) *versus* 35.5% (24.5–56.5), p=0.092; FVC: 76.5±25.7 (4.1) *versus* 65.3±24.8% (4.4), p=0.065).

The 32 patients who had distinct genotypes had a median number of genotypes of 1.3, while the patients with strains belonging to one or more clusters carried a median number of 1.6 genotypes per patient (NS). The 32 patients with distinct genotypes had a median stay duration in the institute of 177 days, *i.e.* during the study period and in the past, while the 44 patients with shared genotypes had a median stay in the institute of 197 days (NS). During the study period, the two groups had a similar duration of stay (group of patients with distinct genotype 52.5 days (35–84) *versus* 81.5 days (40–152) in the group with cluster genotypes, p=0.092).

Among the 44 patients with cluster genotypes, there was one group of 10 patients (including one sibling pair) with the same genotype, one group of seven patients, one of six, two of five, one of four, one of three and six groups of two patients (including two sibling pairs). All three pairs of siblings shared at least one genotype with their sibling. Of the 44 cluster patients, 38 had a shared genotype already on arrival.

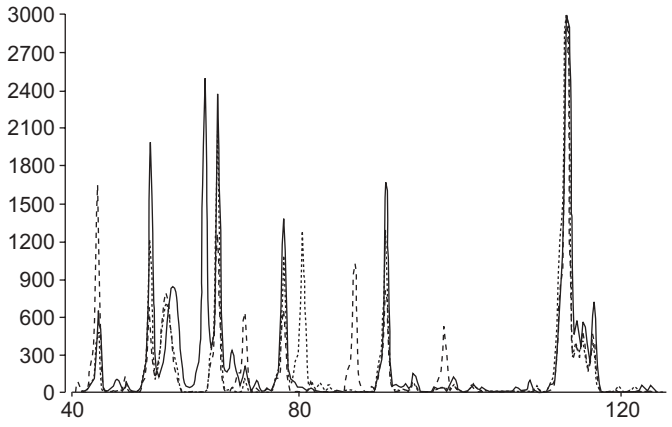

FIGURE 2. Fluorescent amplified fragment length polymorphism fingerprints belonging to three different clusters (—: cluster Y; ·····: cluster U; ----: cluster J) (only fragments between 60 and 120 bp shown).

The median stay during the study of the 68 patients who definitely did not acquire *P. aeruginosa* from another patient during their stay was 60.5 days. Eight patients acquired a new genotype during their stay. The eight patients with new acquisition of a cluster type during the study period (10.5%) had a median stay of 132 days (NS). The null hypothesis of no difference in length of stay between both groups cannot be formally rejected at the 5% significance level. To quantify the size of the difference, a 95% confidence interval was estimated for the difference in median stay between both groups with a bootstrap procedure. This confidence interval was equal to -14,192.5 and is rather wide, but lies largely above 0, indicating a longer stay for the group with possible patient-to-patient transmission.

One patient underwent two episodes of possible patient-to-patient transmission (with two different cluster types), during both study periods. For at least five of these eight patients, the newly acquired genotype, for which a strain with the same genotype was present in another patient during the stay, was transient, since it could no longer be isolated from the patients' sample taken on departure (table 3). Of these eight newly infected patients, three at most were persistently colonised with the newly acquired strain (4%). One patient continued to carry the newly acquired strain 1 yr later, as determined from a nasopharyngeal aspirate taken after lung transplantation. In the other two patients, the newly acquired strain was cultured just before leaving the rehabilitation centre and, unfortunately, no further samples could be obtained thereafter. The patient-to-patient transmission took place twice in the first period and seven times in the second (and longest) study period.

Only four *P. aeruginosa* isolates could be cultured from 13 environmental sampling sites: one from a bedroom sink and three from the recreation rooms. However, these isolates belonged to distinct genotypes and were also different from the patients' genotypes.

DISCUSSION

Debate continues as to whether regular stay in a CF rehabilitation centre is beneficial to CF patients. Benefits

TABLE 3 Numbers of patients with shared genotypes and number of possible patient-to-patient transmissions during the study period

Genotype designation	Patients with this genotype n	Patients with this genotype on arrival n	Patients with shared genotypes with overlapping stay n	Possible patient-to-patient transmission during stay
K	2 (1 S)	2	0	0
M	5	5	0	0
J	2 (1 S)	2	0	0
O	7	5	2	2 T
P	6	5	1	1 P or T
Q	3	2	1	1 P or T
R	2	1	1	1 T
T	4	4	0	0
U	5	3	5	1 P
W	2	0	2	2 T
X	2	2	0	0
Y	10 (1 S)	9	0	1 T
Z	2	2	0	0

S: sibling pair; P: permanent, *i.e.* the same genotype acquired during the stay was still present on departure; T: transient, *i.e.* the same genotype could not be isolated from the patient upon departure.

include opportunity for sport, and intensified and optimised physiotherapeutic techniques. More attention is paid to feeding habits, and the social and psychological benefits of peer contact are obvious. However, some reports have indicated the opportunities for cross-infection of important CF pathogens, in particular *P. aeruginosa* and *B. cepacia* complex species. The aim of this study was to assess the risk of transmission of *P. aeruginosa* in the Belgian rehabilitation centre in De Haan. In Belgium, most CF patients are seen on a regular basis in one of seven hospital reference centres and are sporadically referred to the rehabilitation centre in De Haan, where adult patients live together in a home for several weeks, with separate bedrooms but with shared living and dining facilities. Younger patients live together as in a boarding school. From 1992, segregation has been practiced. Patients are cohorted in a *P. aeruginosa*-negative or a *P. aeruginosa*-positive group during physiotherapeutic sessions and, since 1995, the sinks and water closets are decontaminated daily by alternatively rinsing with vinegar and liquid bleach.

The risk and frequency of *P. aeruginosa* cross-infection among CF patients remains controversial. Genotyping techniques, such as macro-restriction analysis in combination with pulsed-field gel electrophoresis, RAPD and fAFLP have enabled reasonably accurate determination of the clonal relationship of *P. aeruginosa* isolates from patients within a CF centre or region [26–29].

Nevertheless, different conclusions on the transmissibility of this pathogen have been drawn from two recent molecular epidemiological studies from two large centres without segregation policies in Australia [16] and Canada [20]. The Australian cross-sectional study found a widespread clone of *P. aeruginosa* in 55% of 118 infected patients in a paediatric CF clinic [16]. In contrast, the Canadian longitudinal study, run over two decades, showed a low risk of patient-to-patient spread among 174 patients, except for patients with prolonged and close contacts, such as siblings [20]. Previous studies have supported the position of both groups, and indicate the difficulty of making general statements about this highly diverse and adaptable pathogen. Cross-sectional and longitudinal studies in Liverpool [12, 14], Manchester [13, 30, 31] and Sheffield [32] have provided compelling evidence for transmission of highly transmissible strains. In a Norwegian CF centre, 45% of the patients colonised with *P. aeruginosa* carried the same strain [15]; these patients had previous contacts at summer camps and training courses. This once more raised the issue of the risk of cross-infection associated with CF holiday camps. OYENIYI *et al*. [33] previously demonstrated that the five *P. aeruginosa*-negative patients who attended a winter camp in Spain together with 17 patients who were already colonised with *P. aeruginosa* all acquired *P. aeruginosa* strains identical to those carried by the colonised patients. The findings of HOOGKAMP–KORSTANJE *et al*. [34] were completely different. Ninety-one CF patients who attended a CF camp had respiratory cultures performed on arrival, after 2 weeks, after 2 months and regularly thereafter. The incidence of cross-infection was 7% in previously *P. aeruginosa*-negative individuals. The incidence of new and persistent *P. aeruginosa* colonisations was ~2%. The authors concluded that the overall risk of acquisition was comparable to that occurring in the community, and that it was trivial compared with the obvious joy and social benefit derived from a holiday camp. A Brazilian study [35] in an outpatient CF clinic also concluded that the risk of cross-infection is low.

In this study, the genotypic diversity of *P. aeruginosa* isolates was identified initially by RAPD analysis and then with fAFLP in the case of representative isolates of different RAPD types cultured from individual patients. By including multiple isolates from individual patients, the findings of a previous report [26], which concluded that the discriminatory power of RAPD and fAFLP were similar, was confirmed. In all cases studied here, isolates with identical RAPD fingerprints also

had identical fAFLP fingerprints, ensuring that no unrelated isolates were grouped into the same RAPD genotype. For each patient, all of the RAPD products were obtained during the same thermal cycling and electrophoresis run, to avoid differences due to the limited reproducibility of the technique. fAFLP is generally known to be more reproducible and, due to automated digitisation of the fingerprints, it makes large-scale comparison of hundreds of fingerprints possible, an endeavour that is impossible with RAPD analysis. This combined use of a rapid and cheap initial screening technique (RAPD) and a more sophisticated, reproducible and digitised, but also more expensive and laborious technique (fAFLP), enabled the authors to genotype a large number of isolates in an affordable, reasonably convenient, and a highly reliable and discriminatory manner. Moreover, the established library of fAFLP fingerprints of CF *P. aeruginosa* strains can be used for further comparisons and long-term studies.

To the authors' knowledge, this is the first study to examine the risk of cross-infection in a CF rehabilitation centre, where patients live together closely and for long periods of time. Among the 749 *P. aeruginosa* isolates examined, which deliberately included different colony morphology types from an individual patient, only 71 different genotypes were found. In most chronically colonised patients, different colony morphology types were observed on the primary isolation plate, but in most cases these belonged to the same genotype. HOOGKAMP-KORSTANJE *et al*. [34] also observed that isolates dissimilar in macroscopic appearance and of different serotype, pyocin type and phage type, could be of the same, unique genotype. This conclusion was supported by DA SILVA FILHO *et al*. [35].

Forty-nine of the 76 patients (64%) carried only a single genotype, 20 carried two genotypes (26%) and seven carried three types (10%). This confirms the data by MAHENTHIRALINGAM *et al*. [27], who reported that 15 out of 20 patients were colonised by a single strain and that five out of 20 were colonised with two or more strains. This was also in agreement with the findings of HOOGKAMP-KORSTANJE *et al*. [34].

All three sibling pairs in the present study harboured at least one strain in common. These findings are also supported by the data of SPEERT *et al*. [17], who found that 10 out of 12 sibling pairs carried the same strain. GROTHUES *et al*. [36] stated that cross-infection between siblings is common and showed that in three out of the five cases where only one sibling harboured *P. aeruginosa,* the siblings lived in separate homes.

Of the 44 patients that carried a strain that was also present in other patients, 38 already carried this strain on arrival at the CF centre. Therefore, the strain could have been acquired from a common source or from another patient during one of the previous stays in the CF centre, before more stringent infection control measures were introduced. For example, when considering the Y cluster, nine out of ten patients were already colonised with the same strain at the beginning of the study period. Although the 10th patient could have newly acquired his Y-cluster strain (since it could not be cultured from the sample taken at arrival), this strain was isolated sporadically and intermittently from five of the 36 isolates, taken from 16 samples of this patient over a period of 8 months. It is possible that this patient carried the Y-cluster strain at arrival, but only in low numbers. Eight out of the 10 patients with genotype Y isolates attended the centre before the study period and these previous stays overlapped for at least 53 and, at most, 242 days, with a total of 1,583 days of overlap. Therefore, cross-infection could have occurred in this centre during previous stays. However, two children carrying this cluster strain had never stayed in the rehabilitation centre before. It is possible that they acquired this strain at their own CF centres through contacts with other Y-cluster patients.

During the study period, nine episodes in eight patients were noted in which the patient was newly infected by a genotype already carried by another patient during overlapping stay. In such cases it is difficult to avoid the conclusion that patient-to-patient transmission occurred.

The role of the environment as a source of *P. aeruginosa* acquisition in CF-patients is difficult to prove and remains a matter of debate. Most previous studies [16, 37–39] have not been able to identify *P. aeruginosa* in hospital wards or have found only a small number of isolates, which were different from the CF strains. However, DÖRING *et al*. [40] linked several strains from hospital sinks to those carried by patients in sputum, on their hands, throat, nose and anus, and on the hands of staff members. Due to the stringent antiseptic measures, the presence of *P. aeruginosa* in environmental samples at the centre in this study centre may be low. In addition, genotyping showed that those isolates were distinct from those found in patients.

For most patients carrying isolates with the same genotype, it is difficult to assess whether this is due to direct patient-to-patient transmission, to a persistent source of infection in the environment or to continuous recontamination of the environment by colonised patients, which increases the risk of infection from an environmental source. In the De Haan centre, the environment seemed an unlikely source, because the few *P. aeruginosa* environmental isolates that were cultured were different from patient isolates. Furthermore, the large genotypic diversity that one can expect among environmental *P. aeruginosa* isolates would not predict the occurrence of several patients with identical isolates when these isolates had been acquired independently from the environment. Therefore, it seems likely that an important reservoir responsible for *P. aeruginosa* acquisition is the infected CF patient, a hypothesis strengthened by the high number of identical strains found in sibling studies, including this report.

In summary, these findings confirm that different colonial morphotypes of *Pseudomonas aeruginosa* from the same cystic fibrosis patient usually belonged to the same genotype. Since 38 out of the 44 patients with shared genotypes already carried their genotype on arrival, patient-to-patient transmission could have happened in the past, during previous stays. The risk of patient-to-patient-transmission during the study period (with a total stay of 8,218 days) was relatively low (10%), and the risk of persisting colonisation with a newly acquired strain during the study period was also low (4%). The influence of strain-specific differences in *Pseudomonas aeruginosa* transmissibility, infection control practices or acquisition from environmental

reservoirs (natural or contaminated) on the data collected in this study remains unclear. However, it seems reasonable to conclude that each of these factors should be taken into account in debating the controversy that surrounds the prevalence, management and risks of *Pseudomonas aeruginosa* cross-infection.

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ii. Article 2: ***Survey of Pseudomonas aeruginosa genotypes in Belgian colonised Cystic Fibrosis patients***.

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Survey of *Pseudomonas aeruginosa* genotypes in Belgian colonised Cystic Fibrosis patients.

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Genotyping of *P. aeruginosa* from CF patients

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ABSTRACT

To examine whether patients shared genotypes and to compare the genotypes of the isolates from the same patients during two subsequent years, we set up a Belgian databank of P. aeruginosa genotypes of all colonised CF-patients.
Sputum samples from a total of 276 P. aeruginosa colonised patients were sent during 2003 and from a subgroup of 95 patients in 2004. Patients were asked for social contact between each other by questionnaire. All P. aeruginosa isolates exhibiting different colonial morphology on McConkey agar were first genotyped by arbitrarily primed PCR, whereafter single representatives of each RAPD-type were further genotyped by fAFLP-analysis.
For the 213 patients from whom P. aeruginosa could be cultured and 910 isolates, a total of 163 genotypes were found. 75% of patients harboured only one genotype. In most of the limited number of clusters, previous contacts could be suspected. The same P. aeruginosa genotype was recovered from 80% of the patients, studied during both years.

We concluded that most patients harbour only one P. aeruginosa genotype, despite different colonial morphotypes. There is only a limited number of clusters, and most patients seem to have the same genotype during both years.

INTRODUCTION

Pseudomonas aeruginosa, widely spread in soils and water, has been the leading pathogen in cystic fibrosis (CF) lung pathology during the last three decades [1-3]. The means by which this organism is acquired are not yet fully elucidated. After initial infection, chronic infection and colonisation - as defined by the criteria of Döring *et al*. [4] - causes destruction of lung tissue and reduction of lung function, and finally leads to early death. According to the U.S. CF Foundation database of 1996 the median survival of *P. aeruginosa* colonised CF-patients was 28 years, while the median survival for non-colonised patients was 39 years [5]. Kerem *et al.* [6] demonstrated that patients, colonised with *P. aeruginosa* at the age of 7 years, had a mean FEV_1 (Forced Expiratory Volume in one second) that was 10% lower than that of non-colonised patients.

Currently, some CF-centres report the spread of highly transmissible strains that are multi-resistant and, in some patients, are responsible for primary infection [7-12]. Other studies, however, do not find evidence of clonal spread [13-16].

In Belgium the total number of CF patients, registered in the Belgian registry in 2003 [17], was 843, of whom 750 are followed at the 7 CF centres and 280 are considered as colonised by *P. aeruginosa,* according to the criteria of Döring *et al*. [4].

With this study we prospectively set up a Belgian databank of *P. aeruginosa* genotypes, isolated from colonised CF-patients to examine whether patients shared genotypes. In addition we wanted to compare the genotypes of the isolates from the same patients during two subsequent years.

METHODS

Patients

The 7 Belgian CF-centres, Sint Vincentius Hospital/University Hospital Antwerp, HUDERF-Erasme Hospital (ULB) Brussels, University Hospital of the Free University of Brussels (AZ-VUB), University Hospital Ghent, University Hospital Leuven, University Hospital Liège, and Hospital of the Catholic University of Louvain-la Neuve (UCL), participated in this study.

Samples from a total of 276 *Pseudomonas aeruginosa* colonised CF patients, were sent to the microbiology laboratory of the University Hospital of Ghent during 2003.

Sputum samples were mostly collected during an out-patient consultation, at the end of a physiotherapeutic session to ensure that the samples originated from the deeper airways; when patients were too young or unable to expectorate, a nasopharyngeal aspirate or swab was taken (26 nasopharyngeal samples vs. 250 sputum samples). All centres were asked to send another sputum sample one year later. For a subgroup of 95 patients, this sputum sample was obtained.

Patients were aged from 5 to 54 years (with a mean age of 24.2 years ± 8.8 SD).

Patients had to sign an informed consent and approval of the ethics committee of the University Hospital of Ghent was obtained for this national multi-centre study.

Patients filled in a questionnaire (addendum 1 and 2), assessing the frequency and intensity of current and previous social contacts with other CF patients. CF sibling contacts were not taken into account. Scores arbitrarily assigned to the different possible answers in the questionnaire were agreed among the CF specialists involved in the

study, whereby a subjective 'weight' was given to the type of contact (for example an intimate relationship was scored as the highest risk factor for transmission (score 10) and occasional social contact as a minor risk factor (score 4)).

Ninety-three percent of patients, from whom sputum *P. aeruginosa* was cultured, completed the questionnaire.

Segregation policies are installed in all CF centres, except for one centre. In the outpatient clinics, *P. aeruginosa* colonised patients and non-colonised patients are seen on different days. Care givers are strongly advised not to wear jewellery and to wash hands and stethoscopes between each visit. The patients are asked to wash hands before lung function measurement and to produce a sputum sample in a separate room. Filters of the lung function equipment are always changed between patients. Patients with CF are always hospitalised in a single room and contact with other hospitalised CF patients are strongly discouraged.

These recommendations date from the mid nineties and most centres implemented them in the following years.

Microbiology

Sputum samples were inoculated onto McConkey agar (BBL Becton Dickinson, Cockeysville, Md.). After two days of incubation at 37°C, differently looking lactose negative colonies were picked, subcultured on 5% sheep blood agar (BBL) and tested for oxidase. Only oxidase positive colonies were further identified, using tDRNA-PCR [18].

Genotyping

For each patient, all *P. aeruginosa* isolates exhibiting different colonial morphology on McConkey were first genotyped by arbitrarily primed PCR, using alkaline cell lysis for DNA-extraction and Randomly Amplified Polymorphic DNA-fingerprinting analysis (RAPD)

Ready-to-Go beads (Amersham Biosciences AB, Uppsala, Sweden) and primer ERIC2 (AAGTAAGTGACTGGGGTGAGCG) at an annealing temperature of 35°C, as described previously (18). This enabled us to reduce the number of isolates that were subsequently genotyped by the more laborious fluorescent amplified fragment length polymorphism analysis (fAFLP), since only single representatives of each RAPD-type were further genotyped by this procedure. The AFLP-technique is described earlier [19].

Statistical analysis

Values of the 'inter patient contact score' as obtained from the questionnaires did not approach the normal distribution (Kolmogorov-Smirnov Z-statistic $p < 0.001$), therefore all analysis involving questionnaire scores were done under the nonparametric assumption. In order to assess putative differences in number of inter-patient contacts between patients with a unique *P. aeruginosa* genotype compared to patients who share at least one *Pseudomonas* genotype with at least one unrelated patient, median 'inter patient contact scores' were calculated for both groups and compared with the Median test. Dispersion of score values around a median value is presented as interquartile (p25 – p75) ranges. Differences in the distributions of score values between two groups were assessed with the Mann-Whitney U test for two independent samples. Strength of association was expressed as (crude) odds ratios (OR) with 95% confidence intervals (95% CI) to the estimated OR. For any reported measure, statistical significance was accepted, if the two-tailed probability level was <0.05. All analyses were performed with the statistical software package SPSS v. 12.0 (Chicago, Illinois).

RESULTS

P. aeruginosa isolates of a total of 213 out of 276 *P. aeruginosa* colonized patients were genotyped using AFLP-analysis. For 63 patients no *P. aeruginosa* could be isolated, because culture remained negative (n = 10), because technical problems occurred (n = 29) or because another gram negative organism was cultured (n = 24: 6 patients harboured *Achromobacter xylosoxidans* instead of *P. aeruginosa*, 15 patients harboured *Stenotrophomonas maltophilia*, one patient harboured both and 2 patients were colonised with *Burkholderia cepacia*).

A total of 910 *P. aeruginosa* isolates were genotyped using RAPD-analysis.

After excluding isolates from the same patient with an identical RAPD-pattern, 272 isolates from the patients together with an additional 3 reference strains (i.e. epidemic strains from the UK: the Liverpool, Manchester and Midlands strain, kindly provided by Prof. T. Pitt (Public Health Laboratory Services, London, UK) were typed with fAFLP, a technique which is more reproducible than RAPD-analysis and which yields digitized fingerprints allowing large numbers of fingerprints to be compared by computer.

For the 213 patients and 910 isolates, a total of 163 genotypes were found, based on AFLP-analysis. The majority of patients (160) had one genotype, 48 patients had 2 genotypes and 5 patients had 3 genotypes (Fig1).

A limited number of clusters, i.e. 13, with 'cluster' defined as a group of patients carrying *P. aeruginosa* isolates with the same genotype, was observed. There were three additional sibling clusters. The sizes of the clusters and the centre origins are listed in Table 1.

Sixty-six patients (sibling clusters excluded), i.e. 30 %, carried a *P. aeruginosa* isolate with a shared genotype. Five (2.3 %) of them were part of two clusters.

There were six 2-person clusters, one cluster of 4 patients, one of 5 patients, two of 9, two of 10 and one of 12 patients.

Eleven of the 13 non sibling clusters contained patients of multiple centres. In 10 out of 11 multi-centre clusters, former contact between patients could be established, such as stay in a rehabilitation centre (rehabilitation centre A (rehab A) and rehabilitation centre B (rehab B)), attendance to a CF-camp and/or social contact.

When comparing the 'inter patient contact score' between cluster patients and non-cluster patients, there was a significant difference between both groups (100% of the cluster patients filled in their questionnaire versus 90% of the non-cluster patients) (Fig 2). Although mean age of both groups was comparable (24.7 ± 9.1 for the non cluster patients versus 23.2 ± 8.2 years for the cluster group), the cluster patient group (n = 66) reported on average a significantly higher 'inter patient contact score' compared to the non-cluster group (n = 132) (rank-sum $p < 0.001$), i.e. a median inter patient contact score 9.0 (inter-quartile range 5.0 to 14.0) was observed with patients sharing a *P. aeruginosa* genotype with at least one other (unrelated) patient versus a median score of 4.0 (inter-quartile range 0 to 7.0) among patients with a unique *P. aeruginosa* fingerprint ($p < 0.001$).

During 2003, siblings (n = 24) invariably presented with at least one similar *P. aeruginosa* genotype at the level of the sibling pair (n = 12). Siblings represented 4.5% of the non-cluster group of patients (6/132) compared to 27.3% of the cluster group of patients (18/66) ($p < 0.001$), indicating that siblings are actually much more prone to be involved in the spread of *P. aeruginosa* among CF patients (odds ratio = 7.9, 95% CI = 3.0 to 21.0, $p < 0.001$).

The latter observation might be explained by differential inter-patient contact rates, considering that sibling patients (n=24) tended to report on average a higher number of inter-patient contacts compared to unrelated patients (n=174) with median 'inter-patient contact' score values of 5.0 (interquartile range 4.0 to 14.0) and of 4.0 (interquartile range 0.0 to 9.0), respectively, a difference that was marginally significant ($p=0.051$) within the limits of our sample size.

Although inter patient contact scores of siblings may actually be correlated data at the sibling pair level, numbers of colonized siblings were too low in our survey to assess and account for such a correlation and therefore score values of all patients were handled as independent observations. To ascertain however, that a potential interaction at the sibling pair level did not bias our primary results in comparing the score values of the cluster and non-cluster groups of patients, the analysis was also repeated by including only a single value for each sibling pair (a single mean score for each sibling pair, the lowest value of each sibling pair, and the highest value of each sibling pair, respectively) but these analyses did not alter our results.

None of the Belgian *P. aeruginosa* genotypes matched with the 3 UK strains.

For a total of 95 patients, sputum samples were collected from two subsequent years (2003 and 2004) and genotyped by AFLP (see fig.3). In total, the same genotype could be recovered from 76 patients (80%) in both years.

We compared the genotypes that were newly acquired in 2004 with the genotypes already present in the patient's centre in 2003. None of the 'new' genotypes accorded with the known centre genotypes, except for 1 patient, whose novel genotype appeared to be identical to a genotype recovered from his sibling in 2003.

DISCUSSION

This study is, to our knowledge, the first to compare the *P. aeruginosa* genotypes of most colonised CF patients within one country. Most studies examine the variety of genotypes within a centre [12, 14, 20, 21, 22, 23] and do not reflect the national situation.

The only comparable study was carried out in the UK [24], where a national survey was set up to identify and characterize transmissible *P. aeruginosa* strains in CF patients in England

and Wales and for which isolates were requested from over 120 hospitals and a sample size of approximately 20% of the CF population in each centre was attempted, but not always reached.

Different PCR fingerprinting techniques have been developed and different names have been used for identical techniques. Terms such as amplification fragment length polymorphism (AFLP), arbitrarily primed PCR (AP-PCR), DNA amplification fingerprinting or random amplification of polymorphic DNA (RAPD) are often used indiscriminately and create a ' Tower of Babel' phenomenon. In a letter to the editor in the Journal of Clinical Microbiology in 2003 several CF physicians and microbiologists therefore emphasized the need for harmonization of techniques and technique designations for genotyping clinical isolates of *P. aeruginosa* from CF patients [25]. Most of the epidemiological research studies [12, 16, 23] were based on Pulsed Field Gel Electrophoresis (PFGE).
Since our laboratory had already built up experience to genotype other species with RAPD and fAFLP [19], we chose to use these techniques. This choice was supported by a publication of Speijer *et al.* [26], which showed that AFLP analysis was comparable with PFGE and RAPD analysis for *P. aeruginosa* isolates. D'Agata *et al*. [27] also confirmed these findings.

Theoretically, it can be imagined that strains with slightly different fingerprints have acquired only recently mutations which make them differ from each other. As such they may be clonally related, whereas the different fingerprints suggest otherwise. The opposite can be true as well: strains with identical fingerprints can in reality differ from each other, because not all genomic differences are revealed by the fingerprint, which is obtained by looking at only some regions of the genome. When obvious differences exist in parts of the genome that are

not addressed by the technique, these will be overlooked and the technique will yield identical fingerprints for genotypically different strains.

Therefore, it can be stated that genotyping studies are an approximation of the true genetic relatedness among the strains studied. But, whether cross infection is underestimated due to the fact that strains with slightly different fingerprints belong to the same genotype anyway is difficult to say, since overestimation on the other hand is possible as well, as a consequence of genotypically different strains with coincidentally identical fingerprints.

However, the study of Speijer et al. [26], as discussed above, showed that the clustering obtained with three different genotyping techniques (RAPD, AFLP and PFGE), which address different regions of the genome, was concordant, and given the fact that we also found concordance between AFLP and RAPD in this and our previous study [19], we assume that the obtained discrimination reflects the true occurrence of genotypes and that there is neither under- nor overestimation of cross infection.

For the 213 patients and 910 isolates tested , a total of 163 genotypes were found, indicating that different morphotypes in one patient often have the same genotype.

This conclusion is supported by Hoogkamp-Korstanje *et al.* [28] and Da Silva Filho *et al* . [20]. Our previous study [19] in a CF rehabilitation centre also showed that for 76 patients only 71 different *P. aeruginosa* genotypes were found among 749 isolates, indicating that in individual patients isolates with different colonial morphology mostly belonged to the same genotype.

In this national study 75% of the colonized patients carried only one genotype, during 2003. This confirms the data by Mahenthiralingam *et al*. [29] and by our previous study [19] where more than half of the patients (49 out of 76 patients) carried only one genotype, 20 carried two genotypes and seven carried three genotypes.

For 24 out of 279 patients, thought to be colonized by *P. aeruginosa*, another gram negative organism was identified.

Some of the isolates, identified genotypically as *A. xylosoxidans* or *S. maltophilia*, seemed to be considered initially as atypical *P. aeruginosa* in the routine laboratory. Due to the diversity of colonial morphologies and biochemical reactivity encountered, misdiagnosis of gram negative non-fermenters cultured from CF sputum may occur. In one study, misidentification of 11% of *A. xylosoxidans* strains was reported [30].

In our previous report about the occurrence of two large clusters of *A. xylosoxidans* in a CF rehabilitation centre population, the routine hospital laboratory initially also misidentified this organism as *P. aeruginosa* [31].

There was only a limited number of clusters (n = 13 + 3 sibling clusters) and a limited number of patients harbouring one of these *P. aeruginosa* cluster genotypes.

These findings were similar to the data of the Vancouver CF-centre [16], and of the Brazilian study of Da Silva Filho *et al.* [20].

Other centres however reported large clusters with the same genotype. A paediatric CF centre in Victoria, Australia [12] showed that 55% of the 118 *P. aeruginosa* colonized CF children carried the same genotype and the Manchester CF centre [9] had to deal with a multi-resistant strain carried by 14% of its 154 *P. aeruginosa* colonised patients. In the Liverpool CF centre [8] 60% of 92 *P. aeruginosa* colonised children harboured the same strain.

A Norwegian study [11] showed that only 7/60 patients had a distinct genotype, one large main cluster of 27 patients (45%) and remaining clusters of 2 to 4 patients. Patients were known to have contact during holiday camps and training courses.

In the nationwide survey of Scott and Pitt [24], 72% of patients harboured strains with unique genotypes, which matches with our results. In their study small clusters of related strains were evident in some centres, presumably indicating limited transmission of local strains. The most prevalent strain ('Liverpool' genotype) accounted for 11% of patient isolates from 15 of the 31 examined centres. The second most prevalent strain ('Midlands1') was recovered from 86 patients in nine centres and clone C (originally described in Germany) was found in 15 patients from 8 centres. A fourth genotype, identical to the 'Manchester' strain, was found in three centres.

The Liverpool, Manchester and Midlands strain were not detected in the Belgian CF population. Our data did not point to a Belgian 'problem' genotype, carried by many patients, since the largest cluster containing 12 patients (5.5% of the studied population).

We are not aware whether these cluster genotypes are multi-resistant, since susceptibility testing was not performed during this study.

In our study most clusters, i.e. 11 out of 13, contained patients from different CF centres. The vast majority of these patients had spent time in one of the two Belgian rehabilitation centres (rehab A and rehab B), or had participated in a CF camp and at least 2 patients had even shared a hospital room with another non sibling CF patient in the past.

For instance, the largest cluster of 12 patients (cluster 4) contained 6 patients who had stayed several years ago in rehabilitation centre B and 4 patients who had stayed in rehabilitation centre A, whereby the remaining two patients were siblings that had stayed in both centres (for prolonged periods). One could speculate that this sibling pair caused the spread of this cluster genotype. In cluster 6, eight out of the 10 patients previously stayed in rehabilitation centre A. The clustering of the isolates from the remaining 2 patients remains unexplained, since they never stayed there, and since they mentioned close contacts only with

each other, but not with others from cluster 6.

In cluster 8, three of the 5 patients went on a CF holiday camp (the majority of them however could not specify which camp and how long, since these camps took place more than 10 years ago).

One 2-person cluster (cluster 13) contained 2 young school children, followed at the same CF centre: these girls were close friends, and, though they were discouraged to do so, they always came together to the centre, with the same car. They went to the same physiotherapist, shared the same classroom and even wanted to be hospitalised at the same moments. In these two children obviously patient-to-patient transmission had occurred (within the setting of an in- ánd outpatient CF clinic).

Since segregation between *P. aeruginosa* colonised and non colonised patients has been installed in almost all Belgian CF centres (except for centre B) and rehabilitation centres since the mid-nineties, patient-to-patient transmission is suspected to have occurred before that period.

In our previous study in one of the two Belgian rehabilitation centres [19], during 2002 and 2003, 38 of the 45 patients with a cluster strain already carried this strain upon arrival at the CF-centre. Therefore, we could not exclude that acquisition of this strain from a common source, or from another patient, occurred during one of the previous stays in the CF-centre, before more stringent infection control measures were introduced.

That study could establish that the risk of patient-to-patient-transmission during the study period was relatively low (10%), and that the risk of persisting colonisation with a newly acquired strain during the study period was still lower (4%).

In this study siblings carried the same genotype. We did not take into account the sibling clusters, nor did we ask for sibling contacts in the questionnaire, since it could be considered as 'obvious' for siblings to share genotypes [13, 32, 33].

In this Belgian cohort study siblings (total n = 24) represented 4.5% of the non-cluster group of patients (6/132) compared to 27.3% of the cluster group of patients (18/66) ($p < 0.001$) indicating that siblings are actually much more prone to be involved in the spread of *P. aeruginosa* among CF patients (odds ratio = 7.9, 95% CI = 3.0 to 21.0, $p < 0.001$).

We could speculate that siblings stay in a rehabilitation centre more often than non siblings, because the burden of having 2 children with CF (and having to spent a lot of time to their treatment) 'forces' the parents to send their children to these centres from time to time.

It is also possible that siblings are more willing to stay in a rehabilitation centre or to attend a holiday camp, since they don't have to go alone (in contrast with the non sibling patients).

For a subgroup of 95 patients, genotyping was performed for two subsequent years. The vast majority continues to carry its own predominant strain (80%). Of those who had a 'new' genotype in 2004, only one patient had a genotype that matched with a genotype from his own centre in 2003. The strain with the matching genotype however had been isolated from his sister in 2003. Therefore, we could speculate that this strain was already present in the patient during 2003, but had been overlooked, or that it had been newly acquired from his sister in the period between both samplings. Since no other 'new' genotypes in 2004 seemed to match with the 'known centre genotypes' of 2003, we could state that patient-to-patient transmission probably did not occur within the Belgian centres for this subgroup of patients.

A limitation of the study is the lack of validation of the questionnaire. As mentioned in Methods the scores were arbitrarily assigned, since no other scoring system, evaluating the

amount and intensity of social contacts has been used in CF studies.

Although this scoring system remains subjective this questionnaire enabled us to assign to some degree an 'inter patient contact score' to each patient.

In summary, our findings confirm that, in the Belgian CF population, different colonial morphotypes of *P. aeruginosa* from the same CF patient usually belong to the same genotype. We could also state that genotypic diversity among *P. aeruginosa* strains is large in Belgian CF patients. We could describe only a limited number of clusters. The situation is different from one country to another and depends probably on multiple factors such as number of patients per centre, presence of highly transmissible strains, segregation measures. Most clusters in our study could probably be explained by previous social contacts (mostly during previous stays in rehabilitation centres and during holiday camps). Eighty percent of a subgroup of patients continued to carry its own predominant strain during 2 subsequent years, suggesting a small genotype variability in the same patient despite the large genotype diversity in this survey.

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Table 1. *P. aeruginosa* genotypes shared in Belgian CF patients

	Number of patients	Centre							
		A	B	C	D	E	F	G	H
Cluster 1	2		1		1				
Cluster 2	2				1[b]				1
Cluster 3	4			1	1	1		1	
Cluster 4	12				2x2S[c]	6[b]			2
Cluster 5	2					2			
Cluster 6	10	2[b]			2S+1[c]	3			2
Cluster 7	2				1[b]	1			
Cluster 8	5		1		1	3			
Cluster 9	10		2			2x2S+2[c]		2S[c]	
Cluster 10	9		3[b]			2	1	1	2S
Cluster 11	2				1				1
Cluster 12	9	2	1		2S+1[c]	1		2S[c]	
Cluster 13	2				2				

Legend:

[a] Centres A and H form one CF centre on different locations in the same city.

[b] The *P. aeruginosa* strains of one or more of these patients are part of two clusters.

[c] 2S: two siblings or one sibling pair, 2x2S: two sibling pairs, 2S+1: one sibling pair and one unrelated patient.

Fig 1. Flow chart of the study design and of the genotyping

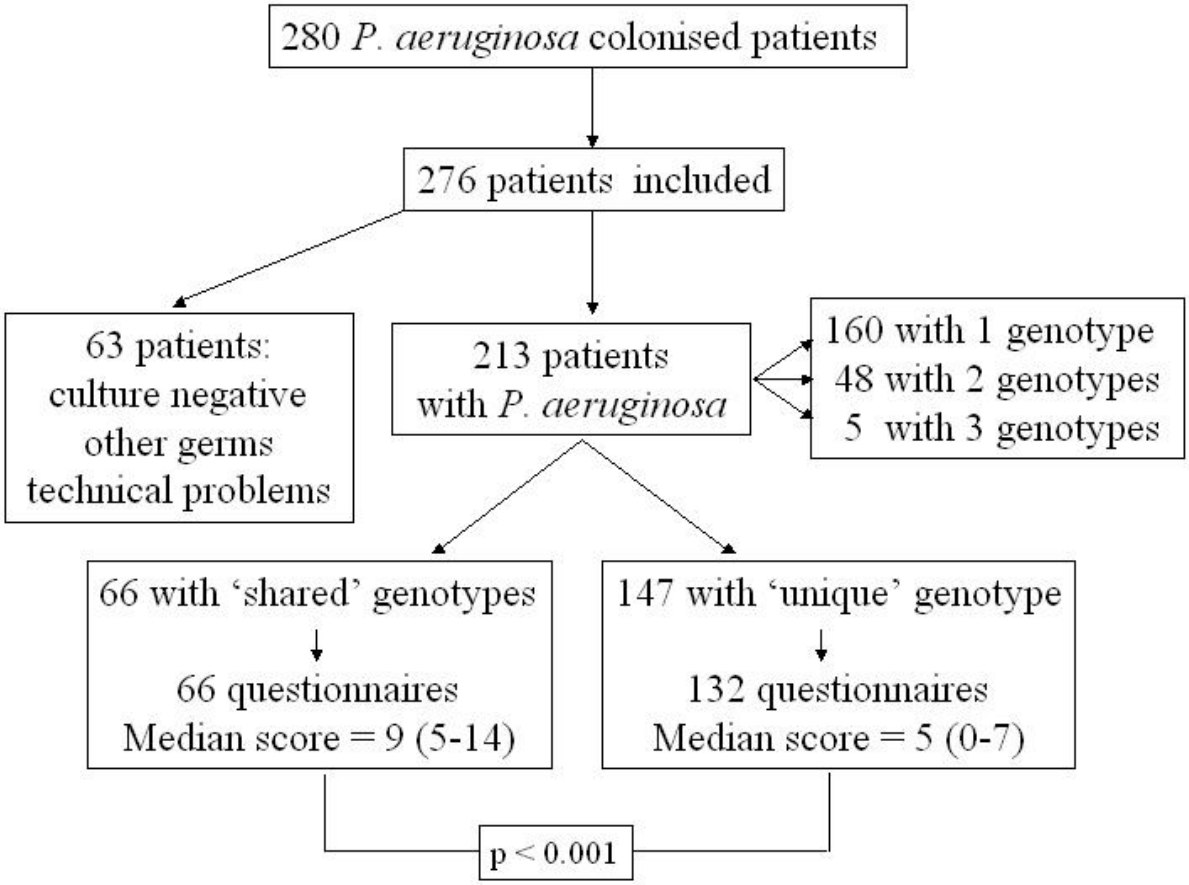


Fig 2. Score of inter patient contacts among patients with a unique *P. aeruginosa* genotype (non-cluster group) compared to that of patients sharing a *P. aeruginosa* genotype with at least one other unrelated patient (cluster group)

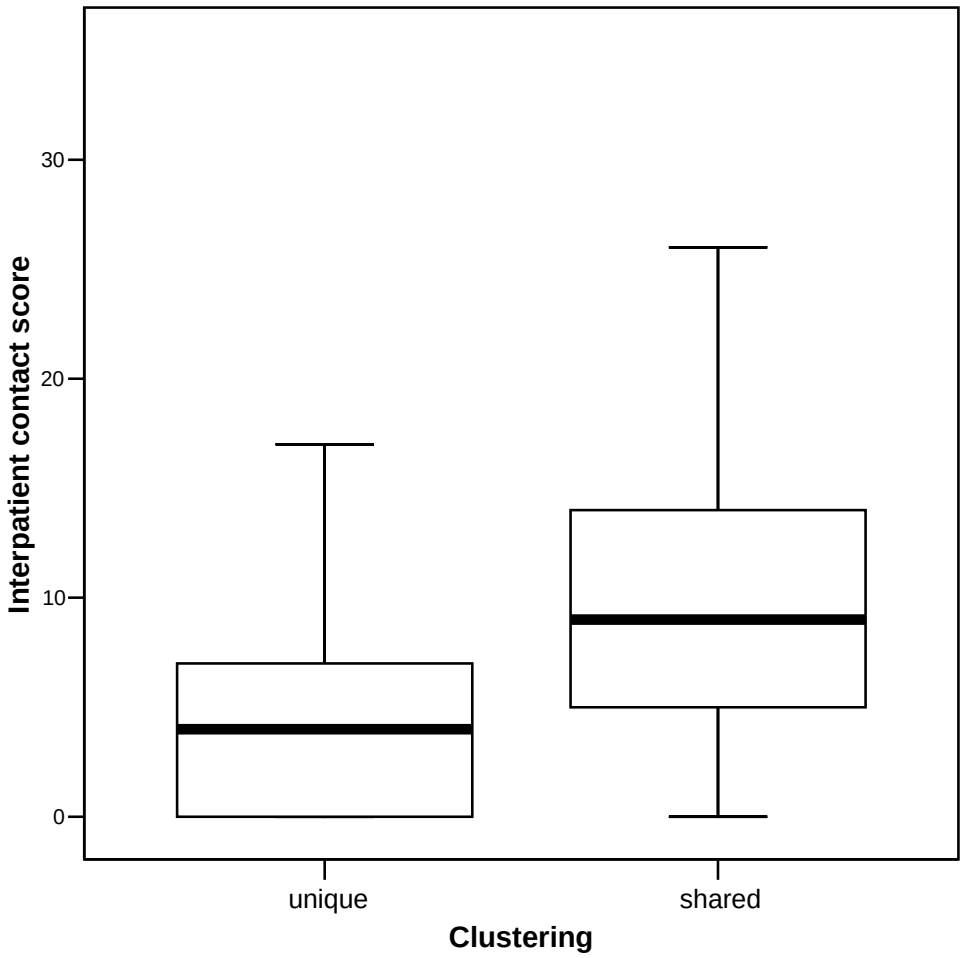
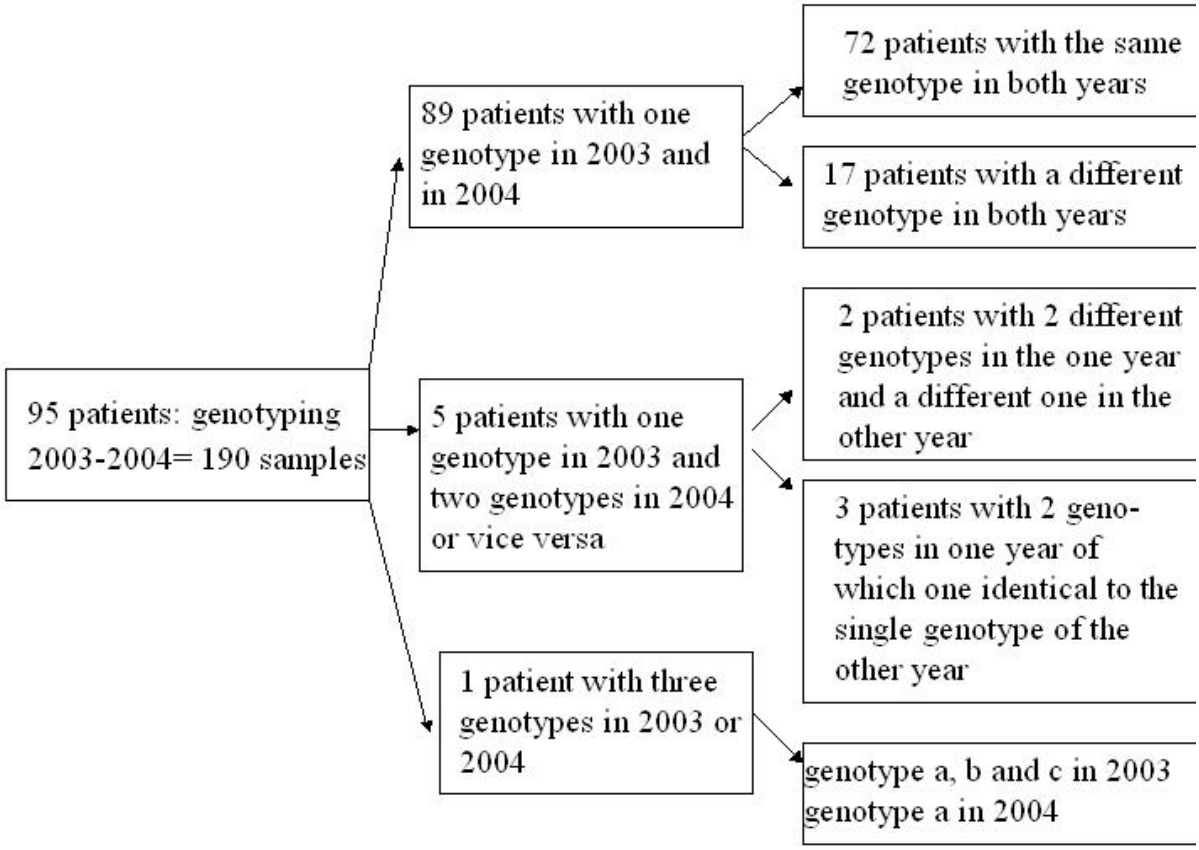


Fig 3. Comparison of genotypes of 95 patients, sampled during both 2003 and 2004



Addendum 1

Questionnaire for parents of CF patients younger than 14 years

1. Does your child have contact with other CF patients in the family, other than brothers or sisters (now or in the past)? YES/ NO

   If yes, with whom?……(initials only) YES = score 8

2. Does your child have contact with other CF patients in the classroom (now or in the past)? YES/ NO

   If yes, with whom?……(initials only) YES = score 7

3. Does your child have contact with other CF patients at school (now or in the past)? YES/ NO

   If yes, with whom?……(initials only) YES = score 4

4. Did your child ever stay in a rehabilitation centre? YES/ NO

   If yes, in which rehabilitation centre and for how long?…… YES = score 5

5. Did your child ever attend a CF camp? YES/ NO

   If yes, which camp and for how long?…… YES = score 5

6. Has your child ever shared a hospital room with another CF patient (brothers and sisters excluded)? YES/ NO

   If yes, with whom?……(initials only) YES = score 5

7. Does your child have social contacts with other CF patients (excluding contacts already asked for in previous questions)? YES/ NO

   If yes, with whom?……(initials only) YES = score 4

TOTAL SCORE=

Remark 1: ‘social contacts’ are ‘physical’ contacts (playing, talking etc.), not written or internet contacts!

Remark 2: you are not obliged to answer these questions, if you don’t want to.

Remark 3: this questionnaire is sent to the investigating lab, under closed envelop.

The members of your CF-centre will not be able to look at the answers.

Addendum 2.

Questionnaire for adolescents and adults with CF

1. Do you have contact with other CF patients in the family, other than brothers or sisters (now or in the past)? YES/ NO

   If yes, with whom?……(initials only) YES = score 8

2. Do you have contact with other CF patients in the classroom (now or in the past)? YES/ NO

   If yes, with whom?……(initials only) YES = score 7

3. Do you have contact with other CF patients at school or at work (now or in the past)? YES/ NO

   If yes, with whom?……(initials only) YES = score 4

4. Did you ever had a sexual relationship with another CF patient? YES/NO

   If yes, with whom?……(initials only) YES = score 10

5. Did you ever stay in a rehabilitation centre? YES/ NO

   If yes, in which rehabilitation centre and for how long?…… YES = score 5

6. Did you ever attend a CF camp? YES/ NO

   If yes, which camp and for how long?……..YES = score 5

7. Have you ever shared a hospital room with another CF patient (brothers and sisters excluded)? If yes, with whom?……(initials only) YES/ NO YES = score 5

8. Do you have social contacts with other CF patients (excluding contacts already asked for in previous questions)? YES/ NO

   If yes, with whom?……(initials only) YES = score 4

TOTAL SCORE =

Remark 1: ‘social contacts’ are ‘physical’ contacts (talking, going out together etc.), not written or internet contacts!

Remark 2: you are not obliged to answer these questions, if you don’t want to.

Remark 3: this questionnaire is sent to the investigating lab, under closed envelop.

The members of your CF-centre will not be able to look at the answers.

c. Transmissibility and clinical impact of *A. xylosoxidans*.

Although colonization of CF patients with *A. xylosoxidans* is well-established, epidemiological studies thus far could not establish much transmission of strains of this species. Dunne and Maisch [121] reported already in 1995 persistent colonization of outpatients with *A. xylosoxidans*, but did rule out patient-to-patient transmission by means of PCR-based genotyping. Vu-Thien et al. [85] found persistent colonization of patients with *B. cepacia*, *S. maltophilia* and *A. xylosoxidans*, but could show the presence of identical isolates in different patients only for *B. cepacia*. In a large study, including 92 A. xylosoxidans positive patients from 46 centres, Krzewinski et al.[86] found five pairs of patients with genotypically identical *A. xylosoxidans* isolates, of which two pairs of siblings and one pair of friends. Furthermore, one additional case of cross-colonization with *A. xylosoxidans* in two siblings was reported [122], persistent airway colonization with *A. xylosoxidans* in two brothers with cystic fibrosis and the presence of the same strain in two of eight colonized children [123]. Recently, Hansen et al. [124] published a study, which showed that 8 of their 16 patients chronically infected with *A. xylosoxidans* harboured a common strain. These findings confirm our data.

Because several authors have indicated that transmissibility of *A. xylosoxidans* is low (with small clusters of maximum 2 patients), we studied the transmissibility of this species among residents of a CF rehabilitation centre.

iii. Article 3: ***Shared genotypes of Achromobacter xylosoxidans strains isolated from patients at a cystic fibrosis rehabilitation centre.***

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Shared Genotypes of *Achromobacter xylosoxidans* Strains Isolated from Patients at a Cystic Fibrosis Rehabilitation Center

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During a study examining transmission of *Pseudomonas aeruginosa* among 76 cystic fibrosis patients in a rehabilitation center, where patients stay in close contact during prolonged periods, several clusters of patients carrying genotypically identical *P. aeruginosa*, as well as two clusters of 4 and 10 patients, respectively, colonized with genotypically identical *Achromobacter xylosoxidans* strains, were discovered.

Clonal spread of *Pseudomonas aeruginosa* strains has recently been reported in United Kingdom and Australian cystic fibrosis (CF) centers (1, 5, 11, 16). This seems to be an emerging infection control problem in CF centers, necessitating the segregation of *P. aeruginosa* colonized and noncolonized patients. A large longitudinal study in British Columbia, Canada, however, did not identify *P. aeruginosa* patient-to-patient transmission in that CF population (21).

Therefore we started a study to determine the prevalence and risk of transmission of *P. aeruginosa* among cystic fibrosis patients in a Belgian rehabilitation center (24). The *P. aeruginosa*-colonized patients lived there together as in a boarding school, with shared dining and living facilities but with separate bedrooms.

During this study we also identified other nonfermenting gram-negative bacilli present together with *P. aeruginosa*. Predominant among these were *Achromobacter xylosoxidans* isolates. Moreover, using randomly amplified polymorphic DNA analysis and amplified fragment length polymorphism (AFLP) typing, we found that several patients were carrying a common genotype of *A. xylosoxidans*.

The taxonomic position of *A. xylosoxidans* has been uncertain during the last decades, leading to name changes from *Achromobacter* to *Alcaligenes* and back to *Achromobacter*. The species was described as the type species of the genus *Achromobacter* (27). Later on, Kersters and De Ley (13) proposed to transfer the type species of the genus *Achromobacter* to the genus *Alcaligenes*. However, more recently, the results of phylogenetic analyses of 16S rRNA nucleotide sequences and a difference of more than 10% in GC content of DNA demonstrated that *Achromobacter xylosoxidans* and *Alcaligenes faecalis*, the type species of the genus *Alcaligenes*, belong to two distinct genera, respectively, *Achromobacter* and *Alcaligenes* (26).

Isolation and identification. Lactose-negative colonies on McConkey agar were isolated on Mueller-Hinton agar containing 5% sheep blood and subsequently tested for oxidase activity. Oxidase-positive isolates were further identified using tDNA-PCR in combination with fluorescent capillary electrophoresis (2). This approach enables us to distinguish between gram-negative nonfermenters such as *P. aeruginosa*, *Burkholderia* species, and *Achromobacter* species (unpublished data) by comparing the tDNA-PCR fingerprints of unknowns with those of reference strains in a library (available at http://allserv.ugent.be/~mvaneech/LBR.html). Identification of isolates as *Achromobacter xylosoxidans* was confirmed by using API20 NE (bioMérieux, Marcy l'Etoile, France). Some of the isolates, identified genotypically as *A. xylosoxidans*, were initially considered atypical *P. aeruginosa* in our routine laboratory. Due to the diversity of colonial morphologies and biochemical reactivity encountered, misdiagnosis of gram-negative nonfermenters cultured from CF sputum may occur. In one study, misidentification of 11% of *A. xylosoxidans* strains was reported (20).

Genotyping. For each patient, the *A. xylosoxidans* isolates were first genotyped by means of arbitrarily primed PCR, using alkaline cell lysis for DNA extraction and randomly amplified polymorphic DNA analysis with Ready-to-Go beads (Amersham Biosciences AB, Uppsala, Sweden) with primer ERIC2 (AAGTAAGTGACTGGGGTGAGCG) at an annealing temperature of 35°C, as described previously (9). For the purpose of selective restriction fragment amplification (AFLP), total bacterial DNA was isolated from fresh cultures on tryptic soy agar by using a QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). AFLP with one fluorescent primer (fAFLP) and with fragment length analysis by means of ABI310 (Applied Biosystems, Foster City, Calif.)-based capillary electrophoresis was carried out basically as described previously (22). Briefly, a combined restriction-ligation procedure was used in which 10 ng of total genomic DNA was incubated with 2 pmol of EcoRI adapter, 20 pmol of MseI adapter, 1 U of EcoRI (Amersham Biosciences), 1 U of MseI (New England Biolabs, Beverly, Mass.), 50 mM NaCl, 50 ng of bovine serum albumin per μl (Roche, Basel, Switzerland), and 4 U of T4 DNA ligase (Amersham Biosciences) in a total volume of 10 μl of 1× reaction buffer for 3 h at 37°C, after which the mixture was diluted 20 times with Tris buffer (Tris [10 mM]–EDTA [0.1 mM], pH 8.0).

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TABLE 1. Adapter and primer sequences used for fAFLP-based genotyping

Adapter or primer	Sequence
EcoI adapter 1	5′ CTCGTAGACTGCGTACC
EcoI adapter 2	5′ AATTGGTACGCAGTCTAC
MseI adapter 1	5′ GACGATGAGTCCTGAG
MseI adapter 2	5′ TACTCAGGACTCATC
EcoRI + 0 primer	5′ (tet)GACTGCGTACCAATTC
MseI + C primer	5′ GATGAGTCCTGAGTAAC

For the selective amplification of the restriction fragments, five microliters of the diluted restriction-ligation mixture was used for amplification in a volume of 10 μl under the following conditions: 0.4 μM TET-labeled EcoR + 0 primer, 1.2 μM *Mse* + C primer (Eurogentec, Seraing, Belgium) (E1), 0.2 mM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1× reaction buffer, and 1 U of GoldStar DNA polymerase (Eurogentec). After 2 min of incubation at 72°C and at 94°C the cycling conditions were 36 cycles of 30 s at 94°C, 30 s at 65 to 56°C, and 60 s at 72°C. During the first 13 cycles, the annealing temperature was lowered by 0.7°C per cycle. After an additional 10 min of incubation at 72°C, the samples were cooled. An overview of PCR primers and adapter sequences is shown in Table 1. To one μl of PCR product were added 12 μl of deionized formamide and 0.3 μl of GS-400 High Density size standard and 0.2 μl of GS-500 size standard, which both contain ROX-labeled fragments in the range of 50 to 500 bp, and this mixture was electrophoresed on an ABI PRISM 310 system (Applied Biosystems, Foster City, Calif.).

A total of 102 *A. xylosoxidans* isolates were cultured from the sputa of a total of 13 patients out of a population of 76 patients studied (Table 2). The sputum cultures of the remaining 63 patients were negative for *A. xylosoxidans*. Only four genotypes were established on the basis of fAFLP genotyping. Two genotypes, designated S and V, were found in 4 and 10 patients, respectively, with 1 patient carrying both genotypes. Therefore, we designated these patients S1 to S3 (patients carrying only genotype S), B (the patient carrying both genotypes), and V1 to V9 (patients carrying only genotype V). Two patients, S3 and B, each had a separate *A. xylosoxidans* genotype in addition to their cluster strains. These genotypes were designated "S3 other" and "B other." Patients V6 and V7 were siblings, carrying not only the same *A. xylosoxidans* genotype V but also identical *P. aeruginosa* genotypes (J). For both siblings, the *P. aeruginosa* genotype (J) was already present at arrival, but the shared *A. xylosoxidans* was acquired after an interval of 6 months (Table 3 and Table 4). Another two patients (V4 and V5), with a common *A. xylosoxidans* genotype (V), also had a *P. aeruginosa* genotype (Y) in common. Only one of the two patients carried the Y genotype at arrival.

Patients B and V2 also shared the same *P. aeruginosa* genotype (U). Patient V2 already carried this genotype at arrival; B acquired it during the fourth month of their overlapping stay. In a previous report (24) we showed that the majority of shared

TABLE 3. Times during study period 1 in which patients stayed at the rehabilitation center

Patient	Patient stay occurred during[a]:							
	January		February		March		April	
	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4
V1								
V2	VU	VU	VU	VU	VU	VU	—	VU
V3								
V4								
V5	—	—	—	—	—	—	Y	
V6	J	J	J	J	J	J	J	JV
V7	J	J	J	J	J	J	J	J
V8								
V9							—	
B	—	—	—	—	—	—	—	U
S1								
S2						S	S	
S3	S	—	S	S	S	—	—	S

[a] Underlining represents the time periods during which patients stayed at the rehabilitation center. Capital letters represent shared genotypes of *A. xylosoxidans* (S, V) and *P. aeruginosa* (J, U, Y).

TABLE 2. Patients and the different genotypes of *P. aeruginosa* and *A. xylosoxidans* isolates

Patient	Total no. of isolates	Total no. of genotypes[a]	No. of isolates of indicated genotype											
			A. xylosoxidans			*P. aeruginosa*								
			S	V	Other	J	I	O	Q	R	T	U	Y	Other
S1	5	1/0	5											
S2	2	1/0	2											
S3	36	2/1	25		2[b]			9						
B	32	3/3	1	1	14[c]							2		1, 13
V1	30	1/1		4										26
V2	63	1/1		24								39		
V3	11	1/1		3							8			
V4	13	1/1		4									9	
V5	37	1/3		7						1			5	24
V6	61	1/1		2		59								
V7	28	1/1		1		27								
V8	16	1/1		6			10							
V9	15	1/3		1					8					2, 4

[a] Number of different *A. xylosoxidans*/*P. aeruginosa* genotypes for this patient.
[b] Designation of the additional *A. xylosoxidans* genotype of patient S3 is "S3 other."
[c] Designation of the additional *A. xylosoxidans* genotype of patient B is "B other."

TABLE 4. Times during study period 2 in which patients stayed at the rehabilitation center[a]

Patient	Patient stay occurred during[a]:																											
	2001												2002															
	September		October		November		December		January		February		March		April		May		June		July		August		October	
	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4	Wks 1 and 2	Wks 3 and 4
V1											V	V														
V2	VU	U	U	U	U	U	U	U	U	U	U		VU				V	VU								—
V3		—									V															
V4						VY	VY	VY	Y	Y	Y	Y	—	—	—	—	—		—	—	—	—	V			VY
V5	Y	Y	V	—						V	V	—														V
V6																			—	J	—	J				
V7		J	J	JV	J	J	J	—																		—
V8			—	V	V	V							V				—			V	—					—
V9			—	—								—			V											
B				—	—	—	—				SV	—	—	—	—					U	—					
S1					S	S																				
S2																										
S3	—	—	S	S	S	S	S	S	S	—	—	—	—	—	—	—	—	—	—	—						

[a] Underlining represents the time periods during which patients stayed at the rehabilitation center. Capital letters represent shared genotypes of *A. xylosoxidans* (S, V) and *P. aeruginosa* (J, U, Y). No new isolations were made during September and the first half of October 2002.

P. aeruginosa genotypes were already cultured at arrival, so patient-to-patient transmission seemed to have happened mostly in the past, before segregation was introduced (since 1992) and before infection control practices such as daily decontamination of the sinks and water closets by alternatively rinsing with vinegar and liquid bleach (since 1995).

The 10 V-cluster patients came from seven different CF centers. During the first study period (from 8 January 2001 until 30 April 2001) (Table 3), 7 of these patients had an overlapping stay: in only one patient (V2) the V genotype was already cultured at arrival and in one patient (V6) a "new infection" could be suspected, and the five other patients remained free of the V genotype during this period. In the second study period (from 1 September 2001 until 23 October 2002) (Table 4), three patients (V2, V4, and V8) already carried the V genotype at (re)admission. However, possibly new infections with the V genotype could be suspected for six patients (V1, V3, V5, V7, V9, and B), all occurring within a period of 6 months (between 1 October 2001 and 1 April 2002).

Three of the four S-genotype patients were followed at the same CF center (B, S1, and S2). The S genotype was already cultured at arrival in three patients (S1, S2, and S3). The fourth patient (B) only showed his S genotype at readmission, after an absence of 3 months. During the previous stays of this patient—lasting 4 and 2 months, respectively—cultures were always negative for the S genotype. It is therefore possible that patient-to-patient transmission took place at the CF center, where the patient was followed with two other S-cluster patients.

All patients colonized with *A. xylosoxidans*, except patients S1 and S2, also carried *P. aeruginosa* genotypes, with patient B having up to three different *P. aeruginosa* genotypes in addition to his/her three *A. xylosoxidans* genotypes. These data at first glance appear to point to a strong tendency of cocolonization with *P. aeruginosa* and *A. xylosoxidans* but may be biased, since the study included only patients assumed to be colonized with *P. aeruginosa*. No study was undertaken to establish in how many cases patients were carrying *A. xylosoxidans* without *P. aeruginosa*. Also, Tan et al. (23) reported that most patients carrying *A. xylosoxidans* were colonized by *P. aeruginosa*, and in another study, six of the eight patients with *A. xylosoxidans* were also colonized with *P. aeruginosa* (17).

We found, for the same population, 14 clusters of *P. aeruginosa*, comprising 2 to 10 patients per cluster (24). Thus, the largest cluster of *A. xylosoxidans* colonization (10 patients) was the same size as the largest *P. aeruginosa* cluster. Furthermore, when a patient was colonized by *A. xylosoxidans*, its isolates always belonged to one of two shared genotypes, with patient B even colonized by isolates of both genotypes, whereas in the same population 45/76 patients (59%) were found carrying a *P. aeruginosa* strain not related to any other strain and thus with a separate genotype. So it can be stated that there was much less genotypic diversity among the *A. xylosoxidans* strains observed compared with the *P. aeruginosa* strains for the same patient population.

A. xylosoxidans has been recognized as an emerging CF pathogen since one study published in 1985 (14) and later on in several others (3, 4, 6, 7, 8). Fabbri et al. (7) identified 12 of the 71 isolates (16.9%) from 24 patients as *A. xylosoxidans*. Ferroni

et al. (8) reported *A. xylosoxidans* to be the second most frequent gram-negative nonfermenter, after *P. aeruginosa*, among 1,093 isolates from 148 patients (10 isolates from 8 patients). Moissenet et al. (17) reported colonization with *A. xylosoxidans* in 6% of 120 CF children, with a mean age of 14.2 years for colonized children. Still, the prevalence may be higher, since in one study *A. xylosoxidans* was detected in CF patients only after usage of a selective medium (18). A phase III study of aerosolized tobramycin showed that a much higher number of the 595 patients were colonized with *Stenotrophomonas maltophilia*, *A. xylosoxidans*, *Aspergillus* species, and oxacillin-resistant *Staphylococcus aureus* than had been established by the CF foundation patient registry efforts (4). In this study, *A. xylosoxidans* (in 8.7% of the patients) was almost as frequently isolated as *S. maltophilia* (10.3%).

Although colonization of CF patients with *A. xylosoxidans* is well established, epidemiological studies thus far have been unable to establish evidence of much transmission of strains of this species. Dunne and Maisch (6) in 1995 had already reported persistent colonization of outpatients with *A. xylosoxidans* but did rule out patient-to-patient transmission by means of PCR-based genotyping. Vu-Thien et al. (25) found persistent colonization of patients with *Burkholderia cepacia*, *S. maltophilia*, and *A. xylosoxidans* but could show the presence of identical isolates in different patients only for *B. cepacia*. In a large study including 92 *A. xylosoxidans*-positive patients from 46 centers, Krzewinski et al. (15) found five pairs of patients, which included two pairs of siblings and one pair of friends, with genotypically identical *A. xylosoxidans* isolates. Furthermore, one additional instance of cross-colonization with *A. xylosoxidans* in two siblings (19) and the presence of the same strain in two of eight colonized children (17) were reported. Our study revealed two large clusters of patients colonized by the same *A. xylosoxidans* strains. These findings are supported by a recent publication of Kanellopoulou et al. (12) that showed that *A. xylosoxidans* isolates of five colonized CF patients were genetically related, suggesting a common-source outbreak.

Although for most of the nonfermenting gram-negative rods the disk diffusion antibiogram is not validated by the CLSI (formerly NCCLS), we carried out an antibiogram to evaluate its possible usefulness as a preliminary typing technique. Several interpretation problems were apparent, such as heterogeneous growth in the inhibition zone and unclear inhibition zone borders. In our hands this approach indicated that all isolates except one showed large inhibition zones for piperacillin (30 to 45 mm) and showed no inhibition zone for amikacin, gentamicin, ofloxacin, ampicillin, aztreonam, temocillin, cefuroxime, and cefotaxime. The activity of ceftazidime and meropenem was very variable, while that of cotrimoxazole and colimycin was difficult to interpret. Fabbri et al. (7) found this organism to be the least susceptible for antibiotics among gram-negative nonfermenting rods and concluded that ceftazidime was most active. We found very variable results for ceftazidime, while for only one strain (B other) no large inhibition zone for piperacillin was observed. Also, Saiman et al. (20) found piperacillin among the most active antibiotics in vitro. There were no consistent susceptibility characteristics which enabled one to differentiate between the four genotypes, but the susceptibility pattern, i.e., colimycin resistance and combined resistance to aminoglycosides and quinolones, was considered to be helpful in distinguishing this species from *P. aeruginosa*.

The pathogenic potential of these newly emerging CF "pathogens" has been ill studied. One study (10) addressed the endotoxic potential of eight species of gram-negative organisms, including *A. xylosoxidans*, and found that, with the exception of *S. maltophilia*, lipopolysaccharide extracted from all of the bacteria upregulated, by various degrees, expression of each of the proinflammatory cytokines assayed. Given the high antibiotic resistance observed in this and previous studies and taking into account that some strains may be transmissible, it may be advisable to pay attention to the presence of *A. xylosoxidans* in the lungs of cystic fibrosis patients. Tan et al. (23) showed in their retrospective case-controlled study of 557 CF patients that the 13 patients that were chronically infected with *A. xylosoxidans* did not deteriorate more in clinical or pulmonary function than patients colonized with *P. aeruginosa* only. More clinical data will be necessary in the future to resolve the issue regarding the pathogenicity of *A. xylosoxidans* in CF patients.

In summary, although several authors have indicated that transmissibility of *A. xylosoxidans* is low, we report the occurrence of genotypically identical strains of this species among two clusters of CF patients attending the same rehabilitation center.

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iv. Article 4: ***A. xylosoxidans in CF: Prevalence and Clinical relevance***

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Achromobacter xylosoxidans in cystic fibrosis: Prevalence and clinical relevance

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Abstract

Background: *Achromobacter xylosoxidans* is increasingly cultured in sputum from cystic fibrosis (CF) patients; nevertheless, there are few published data on the clinical impact of this infection or chronic colonisation.
Methods: Relying on DNA fingerprinting techniques we studied the prevalence of *A. xylosoxidans* in our CF population. In a retrospective case control study the clinical status of patients with at least 3 sputum cultures positive for *A. xylosoxidans* over at least 9 months, at the moment of the first positive culture and during the period of colonisation were compared to age (±1 year), gender and to *Pseudomonas aeruginosa* colonisation controlled CF patients who had never *A. xylosoxidans* positive sputum cultures.
Results: The prevalence of patients with at least one positive *A. xylosoxidans* culture was 17.9%. 5.3% of the patients fulfilled the criteria of our definition of colonisation.

Colonised patients had a median age of 20 years (range 11–27 years) and a mean colonisation period of 1.5 (±0.9) years.

At the moment of the first positive culture we found significantly lower Bhalla scores on HRCT scans of the lungs (11±3 versus 16±3, $p<0.002$), lower Brasfield chest X-ray scores (14±3 versus 18±3, $p<0.019$), lower FVC values (70%±22 versus 94%±12, $p<0.017$) and lower FEV_1 values (55%±32 versus 78%±23, $p=0.123$), although the latter did not reach significance. There was no significant difference in body mass index (BMI) (18.7±3 kg/m^2 versus 19.6±3 kg/m^2, $p=0.8$).

Over the study period *A. xylosoxidans*-colonised patients did have more need for intravenous antibiotic treatment courses (19 versus 5, $p<0.001$); nevertheless, there was no significant difference in lung function decline over the study period (FVC: −6.25±12.34% versus −5.62±8.30%, p 0.77, FEV1: −5.62±8.30% versus −1.87±11.58%, $p<0.47$).
Conclusions: The prevalence of *A. xylosoxidans* infection or colonisation is probably underestimated. Colonised patients are mostly older, with more pronounced lung damage and lower lung function values. Although there was more need for intravenous antibiotic treatment courses, no faster decline in lung function was observed in *A. xylosoxidans* positive patients.

Keywords: Cystic fibrosis; *Achromobacter xylosoxidans*; *Pseudomonas aeruginosa*; Lung function; Morbidity

1. Introduction

Although *Pseudomonas aeruginosa* is the main Gram-negative pathogen found in the sputum of cystic fibrosis (CF) patients, recently other Gram-negative bacilli emerge. Among these emerging pathogens has been *Achromobacter xylosoxidans*. The clinical significance of this micro-organism is unclear and there is limited evidence to direct treatment.

A. xylosoxidans is increasingly cultured in CF sputum; nevertheless, there are few published data on the clinical impact of this infection or chronic colonisation.

In a group of 557 CF patients, Tan et al. [1] reported a prevalence of 2.3%, considering patients with at least three positive cultures over a period of 6 months. In a prospective multi-centre German study Steinkamp et al. [2] reported a prevalence of 1.1% among 1419 CF patients. In the Belgian CF Register 2002 [3], gathering 826 patients, a prevalence of

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1.9% is mentioned, collecting all patients with at least one positive culture over the year 2002. The U.S. Cystic Fibrosis Foundation's National Patient Registry, however, reported over the last 10 years an increase in prevalence of patients harbouring *A. xylosoxidans*: 0.5%, 1.9%, 2.7%, 3.8% and 5.2% in 1995, 1996, 1997, 1999 and 2002, respectively [4].

In order to study the relative risk of cross infection of *P. aeruginosa* in our CF population DNA fingerprinting techniques were carried out on multiple *Pseudomonas* and/or Gram-negative non-fermenting bacilli.

Relying on these DNA techniques we studied the prevalence of *A. xylosoxidans* in our CF population. We evaluated the clinical history of CF patients with at least 3 positive sputum cultures for *A. xylosoxidans* over at least 9 months. In a retrospective case control study we evaluated the clinical status of the patients at the moment of the first positive culture and during the period of colonisation.

2. Materials and methods

A. xylosoxidans is a motile Gram-negative, oxidase-positive rod. The morphology of *A. xylosoxidans* colonies is not that different from that of *P. aeruginosa* colonies.

In our CF centre, taking care of 140 CF patients, *P. aeruginosa* strains and strains of morphologically different looking Gram-negative, non-fermenting bacilli were sent to the DNA laboratory for further identification using DNA fingerprinting techniques.

2.1. Isolation and identification

Lactose negative colonies on McConkey agar were isolated on Mueller Hinton Agar containing 5% sheep blood, and subsequently tested for oxidase activity. Oxidase positive isolates were further identified using tDNA-PCR in combination with fluorescent capillary electrophoresis [5]. This approach enables us to distinguish between Gram-negative non-fermenters like *P. aeruginosa*, *Burkholderia* species and *Achromobacter* species (unpublished data), by comparing the tDNA-PCR fingerprints of unknowns with those of reference strains in a library (available at http://usersallserv.ugent.be/~mvaneech/LBR.html). Identification of isolates as *A. xylosoxidans* was confirmed by using API20 NE (bioMérieux, Marcy l'Etoile, France).

2.2. Patients

A retrospective case control study design was used. In our CF centre each patient is seen on a three monthly basis, when a clinical history is taken, a sputum culture and a lung function measurement are done. In young children or none sputum producers, a pharyngeal swab alternating with a nasopharyngeal aspirate is taken. Patients with at least 3 positive cultures for *A. xylosoxidans* over at least 9 months were compared to subjects who had never grown *A. xylosoxidans*, matched for age (±1 year), gender and *P. aeruginosa* colonisation. Our CF group of 140 patients did not allow to match each colonized patient with 2 control patients, unless we enlarged the age limits to ±2 years which would have weakened our findings because of the large age range (4 years). Comparison was done for chest X-rays and high-resolution CT scans, relying on the Brasfield [6] and Bhalla [7] scores, respectively. Lung function measurements, forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) and body mass index (BMI, kg/m^2) were compared. Over the study period the need for intravenous antibiotic courses and the decline in lung function were evaluated.

Data were compared for the period from the first positive culture for *A. xylosoxidans* until 31 December 2004, giving a mean colonisation period of 1.5 (±0.9) years.

Respiratory function tests were performed on a Masterlab® (Jaeger®).

Respiratory infections in *P. aeruginosa* colonised patients are treated for 2–3 weeks with intravenous antibiotics: an aminoglycoside and a betalactam penicillin. When there was coexistent *A. xylosoxidans* infection antibiotics were chosen, where possible, according to their in vitro activity against both micro-organisms. Elective three monthly intravenous antibiotic treatment policy for chronic *P. aeruginosa* infection is not practised in our CF clinic. Symptomatic patients with positive sputum cultures only for *A. xylosoxidans* were treated, relying to severity, with two intravenous antibiotics chosen according to in vitro sensitivity or with an oral antibiotic (cotrimoxazol or tetracycline) for 2–3 weeks.

Statistical analysis was done using the chi-square test and the unpaired Student's *t*-test for normally distributed data.

3. Results

17.9% of our patient population did have at least one positive culture for *A. xylosoxidans*.

According to our criteria, the prevalence of *A. xylosoxidans* colonisation in our CF-centre was 5.3%. All patients remained colonised with *A. xylosoxidans* throughout the study period.

Eight patients out of 140 at our CF centre were found to be colonised by *A. xylosoxidans*. They had a median age of 20 years (range 11–27 years) and a mean colonisation period of 1.5 (±0.9) years. They were compared to 8 control CF patients, who have never grown *A. xylosoxidans*, matched for age (±1 year), gender and *P. aeruginosa* colonisation.

Seven patients were co-colonised with *P. Aeruginosa*, four by *Staphylococcus aureus* and *Stenotrophomonas maltophilia* was cultured intermittently in 2 patients.

At the moment of the first positive culture we found significantly lower Bhalla-scores on HRCT scans of the lungs (11±3 versus 16±3, $p<0.002$), lower Brasfield chest X-ray scores (14±3 versus 18±3, $p<0.019$), lower FVC values (70±22% versus 94±12%, $p<0.017$) and lower FEV_1 values (55±32% versus 78±23%, $p=0.123$), although the latter did not reach significance. There was no significant

Table 1
Comparison of morphologic and functional parameters at the moment of the first isolation of *A. xylosoxidans* (mean±standard deviation)

	A. xylosoxidans +	*A. xylosoxidans* −	*p*
BHALLA scores	11±3	16±3	<0.002
Brasfield scores	14±3	18±3	<0.019
FVC (% predicted)	70±22	94±12	<0.017
FEV_1 (% predicted)	55±32	78±23	=0.139
BMI (kg/m^2)	18.7±3	19.6±3	=0.8

difference in BMI (18.7 kg/m^2±3 versus 19.6 kg/m^2±3, p=0.8) (Table 1).

Over the study period, *A. xylosoxidans*-colonised patients needed more intravenous antibiotic treatment courses (19 versus 5, p<0.001); nevertheless, there was no significant difference in lung function decline over the study period (FVC:−6.25±12.34% versus −5.62±8.30%, p 0.77, FEV1: −5.62±8.30% versus −1.87±11.58%, p<0.47) (Table 2).

4. Discussion

17.9% of our patient population did have at least one positive culture for *A. xylosoxidans*. The prevalence in our centre was significantly higher than that reported in the literature [1–4]. However, the prevalence measured in our population is cumulative and not annual; moreover, because of an ongoing National *Pseudomonas* study we relied on DNA fingerprinting techniques for species identification. Indeed, some of the isolates, identified genotypically as *A. xylosoxidans*, were initially considered as atypical *P. aeruginosa* in our routine laboratory, using standard phenotypic identification. It is well known that, due to the diversity of colony morphology and biochemical reactivity, misidentification of Gram-negative non-fermenters cultured from CF sputum may occur. In one study, misidentification of 11% of *A. xylosoxidans* strains was reported [8].

The morphology of *Achromobacter* colonies is not that different from the appearance of *P. aeruginosa* colonies. In the routine laboratory where specific mediums or DNA techniques are not available, the true prevalence is probably underestimated.

The prevalence of *A. xylosoxidans* colonisation in our CF-centre was 5.3%.

This is comparable with the findings of Burns et al. [9] who found as part of the pre-enrolment visits for a study on the use of the aerosolised tobramycine, over a period of 6 months, a positive culture for *A. xylosoxidans* on three different occasions in 7% of the 427 screened patients.

As no consensus definition of colonisation is available, we are aware that our definition of colonisation is debatable.

At the moment of the first positive culture we found significantly lower Bhalla scores on HRCT scans of the lungs, lower Brasfield chest X-ray scores, lower FVC values and lower FEV_1 values, although the latter did not reach significance. There was no significant difference in BMI. These findings suggest that particularly patients with more lung damage are prone to infection or colonisation with *A. xylosoxidans*. This could explain the older age at which a first infection is found.

Tan et al. [1] studied 13 patients colonized with *A. xylosoxidans,* with a median age of 17.2 years (range 6.5–32.8). They were compared to 26 control CF-patients matched for gender, age (±2 years), body weight (±10%), FEV_1 (±10%) and bacterial colonisation. Over a period of 4 years they did not find either a significant difference in decrease of lung function parameters, neither significant differences in the use of antibiotics, inhaled antibiotics or oral or inhaled corticosteroids.

As in their study patients were matched for FEV_1, Tan et al. [1] did not look for lung function differences; unfortunately, they neither evaluated HRCT-scan scores, although discrepancy between lung function measurements and morphologic damage, evaluated by HRCT scan scores has been reported [10]. Because they had the opportunity to study a large group of patients, their study would probably have been more informative if they had included all age-matched controls irrespective of their FEV_1.

We chose in our case control study not to stratify for lung function.

This study has the weaknesses of all case control studies.

If one matches the control group for lung function, no conclusions can be made for this parameter as a possible determinant for *A. xylosoxidans* colonisation.

Ideally each colonised patient should be compared with as many controls, matched for age, gender and *P. aeruginosa* colonisation as possible, regardless of their lung function. This would strengthen the findings concerning the possible role of lung destruction as a permissive factor for colonisation and the decline in lung function after colonisation.

A prospective study would of course be more informative; however, it is difficult to predict who will remain culture positive and negative over a certain period, and therefore, large numbers of patients would be required.

Over the study period, *A. xylosoxidans*-colonised patients needed more intravenous antibiotic treatment courses. This finding is not confirmed by the study of Tan et al. [1]. Since in their CF centre patients with chronic *P. aeruginosa* infection received elective three monthly intravenous antibiotic treatment courses, differences between both groups possibly have been attenuated. Whether the higher need for IV antibiotics, as observed in our study, depends on

Table 2
The number of IV AB treatment courses and decline in lung function parameters (mean±standard deviation) over the study period (1.5±0.9 years)

	A. xylosoxidans +	*A. xylosoxidans* −	*P*
IV AB treatment courses	19	5	<.001
Lung function decline			
FVC (% predicted)	6.25±12.34	4.5±11.9	=0.77
FEV_1 (% predicted)	5.62±8.30	1.87±11.58	=0.47

the colonisation by *A. xylosoxidans* or on the more pronounced lung damage remains an unanswered question.

Although there seems to be a tendency, there was no significant difference in lung function decline over the study period. Probably such differences may become evident after a longer follow-up period in a larger group of patients.

Until now, a low transmissibility of *A. xylosoxidans* was reported. However, recently we reported [11] that out of 13 patients colonised with *A. xylosoxidans*, staying in a CF-revalidation centre, 9 patients shared one genotype, three shared another genotype and one patient had both genotypes, suggestive for patient-to-patient spread. Accordingly Kanellopoulou et al. [12] reported 9 colonised patients, 5 of them sharing the same genotype.

Considering the results of Tan et al. [1], who could not detect a need for more intravenous antibiotic treatment courses in *A. xylosoxidans*-colonised patients and our finding that colonised patients have more damaged lungs, it is tempting to hypothesize that *A. xylosoxidans* is a coloniser of more damaged lungs rather than a destructive infectious organism; however, it is obvious that more, especially prospective, studies on the clinical relevance of *A. xylosoxidans* infection or colonisation are warranted.

5. Conclusions

Relying on routine laboratory analysis, the prevalence of *A. xylosoxidans* infection or colonisation is probably underestimated. Mostly older patients, with more pronounced lung damage and lower lung function values have positive cultures. Data on the post-acquisition morbidity showed a higher need for intravenous antibiotic treatment courses. No significantly faster decline in lung function was observed in *A. xylosoxidans* positive patients; however, observations were done retrospectively in a small number of patients over a short period; therefore, one should be cautious interpreting these results. In view of the possibility of patient to patient spread further longitudinal studies are warranted to elucidate the clinical impact of *A. xylosoxidans* infection in CF patients.

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d. Longitudinal analysis of genotypes per patient

While looking for ‘cluster’ genotypes in the studies conducted, we first had to evaluate whether patients harboured one or more genotypes.

In the study in De Haan (p. 73) we followed 76 patients.

From these 76 patients, a total of 749 *P. aeruginosa* isolates, for which the colony morphology on McConkey agar was different, were genotyped by arbitrarily primed PCR (RAPD). For each patient, at least one representative of each different RAPD-type was further genotyped by fAFLP, enabling digital comparison of the genomic fingerprints. Only 71 different *P. aeruginosa* genotypes were found among these 749 isolates, indicating that in individual patients isolates with different colonial morphology mostly belonged to the same genotype. Fifty-seven of these genotypes were only found in a single patient (distinct genotypes), while 14 were found in more than one patient (cluster genotypes). More than half of the patients (49) carried only one genotype, 20 carried two genotypes and seven carried three genotypes.

This confirms the data by Mahenthiralingam *et al*. [103] who reported that 15 out of 20 patients were colonized by a single strain and that five out of 20 were colonized with two or more strains. This was also in agreement with the findings of Hoogkamp-Korstanje *et al* [110]. They observed that isolates dissimilar in colony appearance and of different serotype, pyocin type and phage type, could be of the same, unique genotype. This conclusion was also supported by Da Silva Filho *et al* [111].

In the national study (p. 83) we genotyped *P. aeruginosa* isolates of a total of 213 *P. aeruginosa* colonized patients. For the 213 patients and 910 isolates, a total of 163 genotypes

were found, based on AFLP-analysis. The majority of patients (160) had one genotype, 48 patients had 2 genotypes and 5 patients had 3 genotypes.

This indicated again that different morphotypes in one patient often have the same genotype, thus confirming the data from the ‘De Haan-study’.

For a subgroup of 95 patients, sputum samples were collected from two subsequent years (2003 and 2004) and genotyped by AFLP. Only 6 patients had more than one genotype at one occasion, no patients had more than one identical genotype during both years. Of these 6 samples (on a total of 190 samples, = 95 samples in the 2 consecutive years), 5 contained 2 genotypes and only one contained 3 genotypes. Seventy-two patients had the same single genotype in both years, whereas 17 had a single genotype in both years, differing between both years. Of the 5 patients with 2 genotypes at one occasion, 2 had both genotypes of the one year different from the one in the other year, whereas 3 had one of both genotypes identical to that of the other year. For the patient with 3 genotypes in one year, one of the 3 was identical to the genotype of the other year (Figure 3). In total, the same genotype could be recovered from 76 patients (80%) in both years. In other words the vast majority continues to carry its own predominant strain.

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VIII. Conclusions

In our research on infection/colonization with gram-negative organisms (*P. aeruginosa* and *A. xylosoxidans*) in CF patients, we focused on possible patient-to-patient transmission. With molecular genotyping techniques we tried to answer the questions presented in the chapter V.

- In our first study in the CF rehabilitation centre Zeepreventorium of De Haan we compared the genotypes of the *P. aeruginosa* carried by colonised patients, during several months (**publication 1,** p. 73)

Question 1. Do the patients, chronically infected by *P. aeruginosa* carry a 'unique' or a 'shared' genotype?

Answer: Of the 76 patients observed, 41% were chronically infected by one or more strains with distinct genotypes, i.e. with a genotype unrelated to any other genotype observed in this population, 43% had one or more genotypes shared with other patients, i.e. they carried strains that belonged to a cluster and 16% carried both distinct and cluster strains.

Question 2. Do the patients carry one or more genotypes?

Answer: 49 out of the 76 patients carried only one genotype, 20/76 carried two genotypes and 7 carried three genotypes.

Question 3. Do the patients acquire a 'new' genotype during their stay?

Answer: During the study period 8 patients acquired a 'cluster' genotype. For at least 5 of these 8 patients, the newly acquired genotype, for which a strain with the same genotype

was present in another patient during the stay, was considered as transient**,** since it could no longer be isolated from the patients' sample taken on departure.

Three patients still harboured this newly acquired strain when they left the centre.

Unfortunately 2 patients were lost for follow up (one French and one German patient).In one patient the same strain was cultured one year later, in BAL-fluid, after lung transplantation. In this patient the transmission obviously was persistent.

So we can conclude that patient-to-patient spread had happened during the study period, though infrequently. However, in 58 % of the patients a cluster genotype could be cultured at arrival.This suggests that patient-to-patient transmission could have occurred earlier, and on a larger scale, probably before starting segregation measures, in the mid-nineties.

Question 4. Does patients' genotypes correspond with *P. aeruginosa* genotypes found in the rehabilitation centre environment?

Answer: Only 4 *P. aeruginosa* genotypes could be isolated from the 13 environmental sources sampled, all belonging to distinct genotypes, different from all the patients' genotypes. Thus, infection from the environment seemed unlikely.

- Because of the ongoing debate of the necessity of cohorting patients, chronically infected by *P. aeruginosa*, we set up a national data bank of *P. aeruginosa* genotypes from these CF patients, in collaboration with all 7 Belgian CF centres (**publication 2**, p. 83), to provide answers on the following questions:

Question 5. Do the Belgian CF patients, chronically infected by *P. aeruginosa* carry a 'unique' or a 'shared' genotype?

Answer: 30% of the patients (sibling clusters excluded) carried a *P. aeruginosa* strain with a shared or cluster genotype. Five (2.3%) of them were part of two clusters.

Question 6. Do the patients carry one or more genotypes?

Answer: The majority of patients (160/213) had one genotype, 48 patients had 2 genotypes and 5 patients had 3 genotypes.

Question 7. Do the patients carry the same genotype, when retyped, a year later?

Answer: Sputum of a subgroup of 95 patients(out of 213), chronically infected with *P. aeruginosa,* was cultured again after one year. The same genotype could be recovered in 80% of the patients in both years. The other 20% showed at least one different genotype at both culture moments. Only one patient showed in the second year a new ' shared' genotype, already present in his sibling the year before.

Question 8. When patients share the same genotype, is there a correlation with the amount and intensity of social contact?

Answer: When comparing the ‘inter patient contact score’ between cluster patients and non cluster patients, there was a significant difference between both groups. Although mean age of both groups was comparable (24.7 ± 9.1 for the non cluster patients versus 23.2 ± 8.2 years for the cluster group), the cluster patient group reported on average a significantly higher ‘inter patient contact score’ compared to the non-cluster group (rank-sum $p < 0.001$), i.e. a median inter patient contact score 9.0 (inter-quartile range 5.0 to 14.0) was observed with patients sharing a *P. aeruginosa* genotype with at least one other (unrelated) patient versus a median score of 4.0 (inter-quartile range 0 to 7.0) among patients with a unique *P. aeruginosa* fingerprint ($p < 0.001$).

- During the "De Haan" study we observed frequent co-colonisation with *A. xylosoxidans* . Since there is little knowledge about the transmissibility of this organism within CF patients, we wanted to answer the following questions (**publication 3,** p. 113)

Question 9. Do the patients, chronically infected with *P. aeruginosa,* who are co-infected or even co-colonised with *A. xylosoxidans* carry a 'unique' or a 'shared' genotype of the latter organism?

Answer: On a total of 13 patients only four genotypes were established based on fAFLP-genotyping. Two genotypes, designated S and V, were found in four and ten patients respectively, with one patient carrying both genotypes. Two patients each had a separate *A. xylosoxidans* genotype in addition to their cluster strains. This indicated that there was little genotypic variation and patient-to-patient transmission likely occurred in the past, since for a total of 102 isolates of 13 patients only 4 genotypes could be found! Recently the group of Hoiby [124] published their data, which showed that 8 of their 16 patients chronically infected with *A. xylosoxidans* harboured a common strain. These findings confirm our data.

Question 10. Do the patients carry one or more genotypes of *A. xylosoxidans*?

Answer: 10 out of the 13 patients carried one genotype, 3 carried 2 genotypes.

Question 11. Do the patients acquire a 'new' genotype of *A. xylosoxidans* during their stay?

Answer: Six of the 13 patients acquired a cluster genotype of *A. xylosoxidans* during the study period (indicating patient-to-patient transmission during the study period).

Seven patients already carried their cluster strain at (re)admission. Most of these 7 patients had previous stays in the rehabilitation centre or were followed in the same CF centre so, for these patients, patient-to-patient transmission seemed to have happened in the past. Previous studies [85, 121] failed to demonstrate such large clustering, but in both studies only 8 CF patients with *A. xylosoxidans* were considered. Krzewinski et al. [86] however included the patients of 69 American CF centres, and ended up with 92 *A. xylosoxidans* positive patients in 46 centres with 5 instances of shared genotypes (2 siblingpairs and 1 pair of friends). Segregation policy is however not described in this study.

None of these studies were performed in a rehabilitation centre. Perhaps the close contacts in such a "resident" setting have favored the patient-to-patient transmission. Of course this study only includes a small amount of *A. xylosoxidans* colonized patients, thus conclusions should be interpreted carefully. Larger studies are therefore warranted.

-The clinical significance of *A. xylosoxidans* in CF remains unclear and until now, there is limited evidence for necessity of treatment.

Therefore we set up a retrospective case control study to answer the following questions (**publication 4**, p. 120):

Question 12. What is the prevalence of *A. xylosoxidans*(= at least one positive culture with *A. xylosoxidans*) in our CF centre population and what is the prevalence of *A. xylosoxidans* colonisation (= at least 3 positive cultures during a period of 9 months) in our CF centre population?

Answer: The prevalence of at least one positive *A. xylosoxidans* culture in our CF centre population is 17.9% and the prevalence of *A. xylosoxidans* colonisation in our CF centre is

5.3%. Colonisation or chronic infection was especially seen in older patients, with more pronounced lung damage.

Question 13. What is the clinical impact of *A. xylosoxidans* colonisation?

Answer: Over the study period *A. xylosoxidans* colonized patients needed more intravenous antibiotic treatment courses (19 versus 5, $p < 0.001$), nevertheless there was no significant difference in lung function decline over the study period (FVC: -6.25% ± 12.34 versus - 5.62% ± 8.30, $p = 0.77$, FEV1: -5.62%± 8.30 versus -1.87%± 11.58, $p < 0.47$).

From all these results we conclude that the diversity of *P. aeruginosa* genotypes in the Belgian CF population is large, although cluster genotypes are present. For *A. xylosoxidans* the genetic diversity in the rehabilitation centre is limited.

Several of our findings point to the importance of patient-to-patient transmission :

1. ***P. aeruginosa* cluster strains are present in the Belgian population (although infection from a common environmental source can not be excluded).**
2. **transmission of *P. aeruginosa* during the study period in the rehabilitation centre of De Haan is confirmed, although the transmission is mostly transient.**
3. **siblings almost always had the same *P. aeruginosa* genotype.**
4. **in the national study patients with a shared *P. aeruginosa* genotype had a significantly higher inter patient contact score.**
5. ***A. xylosoxidans* cluster strains are present in the rehabilitation centre population , moreover, all patients carrying *A. xylosoxidans* shared at least one strain with**

others. We could even show that 2 patient pairs with the same *A. xylosoxidans* also shared the same *P. aeruginosa* genotype.

Thus, this work in general supports the segregation measures for CF patients, so much debated in CF literature.

IX. Perspectives for further research

Derived from the experience we built up the last years in the domain of genotyping of these gram-negative organisms in CF, other research projects have been set up.

A national multicentre study is almost completed, examining the possible home environmental origin of *P. aeruginosa,* when the germ is isolated for the first time from sputum of a formerly *P.aeruginosa* negative patient. .

The 7 Belgian CF-centres send sputum of 'newly' infected *P.aeruginosa*. patients between Jan. 2003 and Dec. 2005. The CF-nurses took cultures at the home of the patient (sinks, showers, swimming pools, nebulizers etc…). The environmental ánd patient isolates will be compared genotypically and the patients 'newly acquired ' *P. aeruginosa* genotype will be compared to the 'Belgian database', to study whether patient-to-patient transmission occurred.

Also another multi-centre study will be started soon, investigating the use of molecular methods for early detection of *Pseudomonas aeruginosa* infection.

Although early infection with *P. aeruginosa* is aggressively treated with increasing success there are currently no treatment schedules which enable eradication of *P. aeruginosa* once colonisation is established. Therefore early treatment can significantly increase quality of life and life expectation.

Early treatment, however, requires means for early detection of infection.

In order to detect early infection by *P. aeruginosa*, regular sputum cultures, pharyngeal swabs or nasopharyngeal aspirates are carried out at all Belgian CF centres. At the moment of the first positive culture for *P. aeruginosa* one wonders for how long the micro-organism was already in the lungs of the patient. Therefore, since it is of utmost importance to start antimicrobial treatment as early as possible and as such enhance the possibility to eradicate *P.*

aeruginosa, more sensitive detection methods are warranted. Thus, PCR-techniques on sputum, nasopharyngeal aspirate and broncho-alveolar lavage will be developed and compared with cultures. PCR positive/culture negative patients will be followed intensively and time to a positive regular culture will be evaluated. The PCR results will be correlated with a dosage of *P. aeruginosa* antibodies.

Summary

Pseudomonas aeruginosa is known to be the most important pathogen in CF, and is associated with increased morbidity and reduced life expectancy. Although we are convinced that peer contacts are psychologically beneficial for patients dealing with CF, we wanted to ensure that our patients did not experience more harm than benefit from these contacts, by patient-to-patient transmission of bacteria. Therefore, we set up 2 studies to evaluate the risk of patient-to patient transmission: a pilot study in the rehabilitation centre of De Haan (2001-2002) and a national study, in collaboration of all 7 Belgian CF centres (2003-2004). From both studies we could derive that the majority of *P. aeruginosa* colonised patients carried a unique genotype, though small clusters of patients (ranging from 2 to 12 patients) with the same genotype could be described. During the study period in the rehabilitation centre, where patients stayed for at least 3 weeks, there were 3 patients for whom persistent cross-infection could be suspected. We considered this risk comparable with the risk of getting infected from the environment. Both studies showed that, in the individual patient, morphologically different looking colonies often had the same genotype. The national study revealed that most patients (80%) continue to carry the same strain, when genotyped one year later. For those who had a different genotype than the year before, no 'new' genotypes could be linked with the known centre genotypes of 2003, except for 1 patient, whose novel genotype appeared to be identical to a genotype recovered from his sibling in 2003. This made us draw the conclusion that the segregation policy in the CF centres seems to work!

It also showed us, by means of a questionnaire score, that the patients carrying a 'cluster'genotype, had a significantly higher score for social contacts than those with a 'unique' *P. aeruginosa* genotype. This led to the conclusion that the 'cluster'genotypes are acquired from patient contacts in the past, which supports the need for segregation. These

studies also showed that about 2/3 of the *P. aeruginosa* colonised patients carried only one genotype, about 1/3 2 genotypes and only a small minority 3 types.

We also described probable patient-to-patient transmission of *A. xylosoxidans* in the rehabilitation centre population, with only four genotypes that were identified from 102 isolates of 13 patients. These findings contradicted previous studies reporting that transmissibility of *A. xylosoxidans* is low! *A. xylosoxidans* is an emerging pathogen in older CF patients.
Because litlle is known about the transmissibility and pathogenicty of the organism, the need for segregation *A. xylosoxidans* colonised patients remains unclear.
Therefore we studied the prevalence of *A. xylosoxidans* in our CF population and the possible pathogenicity of this organism in a retrospective case control study. At the moment of the first positive culture we found significantly lower Bhalla-scores on HRCT scans of the lungs, lower Brasfield chest X-ray scores and lower FVC values. There was a significantly higher need for intravenous antibiotics for *A. xylosoxidans* colonized patients, but no more rapid decline in lung function. Therefore we hypothesized that *A. xylosoxidans* is a coloniser of more damaged lungs rather than a destructive infectious organism, however it is obvious that more, especially prospective, studies on the clinical relevance of *A. xylosoxidans* infection or colonisation are needed.
The low prevalence of *A. xylosoxidans* (and *S. maltophilia)* in individual centres warrants national or even international studies to draw statistically significant conclusions about the pathogenicity and need for treatment of these organisms.

Samenvatting

Pseudomonas aeruginosa is het belangrijkste pathogeen micro-organisme bij mucoviscidosepatiënten, en leidt tot verhoogde morbiditeit en verminderde levensverwachting. Niettegenstaande onze overtuiging dat contacten tussen patiënten onderling psychologisch voordelig kunnen zijn, wouden we er ons toch van vergewissen dat deze contacten niet meer kwaad dan goed deden, door mogelijks kruisinfectie.

Daarom hebben we 2 studies opgezet om dit risico op kruisbesmetting te evalueren: een pilootstudie in het Zeepreventorium van De Haan (2001-2002) en een nationale studie, in samenwerking met de 7 Belgische CF-centra (2003-2004).

Uit beide studies konden we concluderen dat de meerderheid van de patiënten een 'uniek' genotype van *P. aeruginosa* droegen, hoewel (eerder kleine) 'clusters' van patiënten (2-12 patiënten) met hetzelfde genotype konden beschreven worden.

Tijdens de studie in het revalidatiecentrum van De Haan, waar patiënten minimum 3 weken verbleven, kon voor 3 patiënten een persisterende kruisinfectie vermoed worden. We beschouwden dit risico vergelijkbaar met het risico om uit de omgeving besmet te worden. Beide studies toonden aan dat colonies van *P. aeruginosa*, die er op de kweekplaat fenotypisch verschillend uitzagen, uiteindelijk vaak genotypisch gelijk waren.

De nationale studie leerde ons dat patiënten meestal hetzelfde genotype droegen, één jaar later. Wanneer echter een ander genotype gevonden werd 1 jaar later, kon dit niet gecorreleerd worden aan gekende genotypes uit 2003, behalve bij 1 patiënt, van wie de broer dit genotype reeds droeg in 2003. Daaruit durfden we concluderen dat de segregatiemaatregelen in de centra wel degelijk hun werk doen.

Aan de hand van een vragenlijst die polste naar verschillende mogelijkheden van sociaal contact onder patiënten, konden we ook aantonen dat patiënten met een 'cluster'type een hogere 'contact score' hadden dan de patiënten met een 'uniek' genotype.

Dit leidde tot de conclusie dat de 'clustergenotypes' verworven werden via kruisinfecties in het verleden (vóór de segregatiemaatregelen van toepassing werden), wat ons steunde in onze overtuiging dat segregatie tussen *P. aeruginosa*-positieve en -negatieve patiënten een noodzaak is.

Beide studies toonden ook aan dat ongeveer 2 derden van de gekoloniseerde patiënten slechts 1 genotype 'huisvesten', ongeveer 1 derde 2 types en slechts een kleine minderheid 3 types.

We beschreven ook mogelijke kruisinfectie van *A. xylosoxidans,* gezien tijdens de studie in het revalidatiecentrum van De Haan uit 102 isolaten van 13 patiënten (met co-colonisatie van *P. aeruginosa*) uiteindelijk maar 4 verschillende genotypes konden worden gevonden. Deze bevindingen spraken vroegere literatuurgegevens tegen, die stelden dat kruisinfectie met *A. xylosoxidans* weinig waarschijnlijk was.

Er is echter nog maar weinig gekend over het ziekmakend vermogen en de transmissie van dit organisme, vandaar dat er geen standpunt bestaat over de nood van segregatie bij *A. xylosoxidans* gekoloniseerde patiënten. Daarom bestudeerden we de prevalentie van *A. xylosoxidans* in onze CF populatie en de pathogeniciteit in een retrospectieve 'case control' studie. Deze studie toonde aan dat op het moment van eerste positieve kweek, er reeds een significant lagere Bhalla-scores op longCt wordt gezien, evenals lagere Brasfield scores van de conventionele RX thorax en lagere FVC worden.

Ook werd aangetoond dat de patiënten met *A. xylosoxidans* een hogere nood aan intraveneuze antibioticakuren hadden, doch géén snellere longfunctievermindering. Vandaar de hypothese dat *A. xylosoxidans* eerder een 'colonisant' is van sterk beschadigde longen , dan wel een echt ziekmakend organisme. Het is duidelijk dat er meer studies nodig zijn, en dan liefst prospectieve, om deze zaak op te helderen.

Om de zorg voor de mucoviscidosepatiënten nog verder te verbeteren moeten er nationale, of nog beter, internationale studies opgezet worden, gezien individuele centra over een te klein aantal *A. xylosoxidans (*én *S. maltophilia)* gekoloniseerde patiënten beschikken om statistisch significante conclusies te trekken over de pathogeniciteit, de behandeling én eventuele preventiemaatregelen van deze kiemen.

Curriculum Vitae

Sabine Van daele werd geboren op 23 mei 1964 te Gent.

Na het behalen van haar ASO diploma Latijn-Grieks, aan het Sint-Bavohumaniora te Gent in 1982, studeerde zij geneeskunde aan de Rijksuniversiteit Gent (1982-1989). Tijdens haar studies werkte zij op het Anatomopathologisch laboratorium o.l.v. Prof. Dr. Roels en op het laboratorium voor Genetica o.l.v. Prof. Dr. F. Speleman.

Het diploma van Doctor in de genees-, heel- en verloskunde werd in 1989 met grote onderscheiding behaald.

Van 1989 tot 1994 werkte zij als assistent Pediatrie in de Universitaire Kinderkliniek Gent.
Na haar erkenning als kinderarts werd zij aangesteld als resident op de dienst Kinderlongziekten o.l.v. Prof.Dr. F. De Baets.
In 2000 werd zij adjunkt-kliniekhoofd.
De klinische activiteiten werden aangevuld met een onderzoeksmandaat van het Fonds voor Wetenschappelijk Onderzoek – Vlaanderen in 2001-2002. Dit onderzoek naar mogelijke kruisinfecties bij mucoviscidosepatiënten die verbleven in het Zeepreventorium van De Haan gaf de aanzet voor de verdere uitbouw van het CF-onderzoek.

Zij nam de laatste jaren deel aan verschillende onderwijsopdrachten (begeleiden E-lijn 2de kand. en 1ste proef Geneeskunde sinds 2001/2002, tutorials Hart-Nier-Long 2de kand. Geneeskunde sinds 2001, klinische lessen 2de proef, Interuniversitair onderwijs kinderpneumologie (jaarlijks) en postgraduaat kinderpneumologie (interuniversitair) (2-jaarlijks)).

Tussen 1999 en 2004 was zij lid van BRIC (Belgian Respiratory and Infectiology Commitee), vanaf 2001 is zij lid van het bureau 'Belgische Kring voor KinderLongartsen '(BKKL), tussen 2003 en 2005 was zij secretaris van deze vereniging.

Sabine Van daele is auteur en co-auteur van meerdere nationale en internationale publicaties.

Zij is getrouwd met Jo Müller en heeft 3 kinderen: Arno, Zoë en Eva.

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